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Emerging Trends in Dietary Components for Preventing and Combating Disease

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

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Preface

Agricultural and food derived natural products (defined here as secondary metabolites produced by living organisms) have played an important role in human health promotion and disease prevention for centuries. Approximately 47% of the drugs approved by FDA during 1981-2006 were originally derived from natural products. World's Health Organization (WHO) estimated, nearly 80% of the consumers in the world, utilize plant products as their first line of defense against diseases and as their primary sources of medicines. Recent research from several laboratories around the world has clearly demonstrated that several of these agricultural and food derived products have profound impact on human health. Four interdisciplinary scientists including Horticulturist, Natural products chemist and Pharmacologists have taken a pivotal role to address this issue by organizing the symposium.

The collection of book chapters presented here are selected from a special symposium entitled "***Agricultural and Food Derived Natural Products for Preventing and Combating Disease***" organized by the co-editors at the recent 240th American Chemical Society National Meeting, August 22−26, 2010 at Boston, MA. At this meeting, 47 international scientists from 14 different countries with common interests were successfully brought together in this symposium to exchange the ideas and selected book chapters were published.

This book provides an integrated approach to address the chemistry of natural products for their application in disease prevention through in vitro, animal and human intervention studies. This book covers three main areas: 1) Purification and characterization of certain natural compounds, 2) *In vitro* models for prevention and combating disease, 3) *In vivo* and human intervention models for prevention and combating diseases.

The first area focuses on chemistry of natural products with studies involving isolation methods and elucidation of structural properties of natural compounds including polymethoxyflavones, flavonoids, furocoumarins, limonoids, polyacetylenes, procyanidins, cardiac glycosides from various fruits, vegetables and medicinal plants. Correlation studies of natural extracts from berry fruits along with their chemical biological responses are described. Metabolic profiling using NMR spectroscopy methods for disease diagnostics provides a comprehensive characterization approach for further understanding the role of natural products. Following up on the chemistry, *in vitro* models for preventing and combating disease are presented. *In vitro* models involving products derived from olives, hawthorn, goji, carrot, citrus, berry, and sugar maple provide interesting information on their probable role in disease preventing properties. In addition to traditionally cultivated, several indigenous plants from different regions of the world such as Chilean wild plants, Mediterranean wild plants, South east Asia, Taiwan and Jeju provide an understanding of their importance in development of healing medicines. Recent advances in *Azadiracta indic*a, and *Echinacea pallida* will enhance the knowledge and further implicate their role as a promising medicinal plant species. Third and fourth areas include *in vivo* models and human intervention studies for prevention and combating diseases. These chapters compliment the chemistry and *in vitro* sections by imparting an overview on the future applicability of the natural products. Results from studies on anthocyanin based fruits and vegetables including berries, natural pigments, sesamol, Okra seeds, Grape seed extract, Methyl jasmonate will be presented to give justification of their health benefits through modulation of different metabolic pathways. The book will contain all of the basic information presented at the symposium.

Given that the worldwide incidence of heart disease, cancer and other chronic human illnesses is rapidly increasing, intervention with agricultural and food derived natural products, provide an attractive strategy for disease prevention. Therefore, this is book provides focused and timely discussions will create a great interest to both basic and clinical researchers as well as other health care professionals. We believe this book will facilitate further research in this area.

The editors acknowledge all authors for their patience, hard work and their timely contributions as well as rest of the speakers who gave oral presentations in four day symposium at Boston. Further, we wish to thank deeply reviewers for their critical suggestions/comments for making this book possible. Editors also acknowledge Mr. Rammohan Uckoo, Graduate student, VFIC, for his technical help. Finally, we would like to thank ACS Books Division, Bob Hauserman, Tim Marney, Kat Larsen as well as individuals from press especially Mary Calvert and Pamela Kame, for publishing this symposium series.

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Chapter 1

Chromatographic Techniques for the Separation of Polymethoxyflavones from Citrus

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Polymethoxyflavones (PMF's) are naturally occurring flavonoids present in Rutaceae family in the Citrus genus. Due to the potential use of PMF's as a chemopreventive agent and functional food, a rapid reproducible method for large scale isolation is critical. Isolation of PMFs from citrus is a challenging task due to complex sample matrices. Recent advances in chromatographic techniques led to several isolation and identification methods of PMF's from citrus. The present chapter discusses about the various techniques used for extraction and isolation of PMFs from different citrus species. The identification of these compounds using ^{1}H NMR and ^{13}C NMR is also described.

Introduction

Citrus is one of the most consumed and cultivated fruit crop. Apart from its savory taste, citrus consumption is also correlated with various health benefits due to the presence of several bioactive compounds. Some of these bioactives include limonoids (*1*, *2*), flavonoids (*3*, *4*), carotenoids, phenolic acids (*5*), organic acids (*6*), furocoumarins (*7*, *8*), and amines (*9*). Due to rapid hybridization and mutations coupled with polyploidy nature of citrus led to development of several varieties. These genetical variations might have resulted in characteristic changes in the levels of bioactive compounds. Based on the levels of bioactive compounds, chemotaxonomy was also proposed for the classification of the citrus genus based on the variation of limonoids (*10*). Similarly, the variation in composition of

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polymethoxyflavones was also used as a basis for chemotaxonomic classification of the Citrus genus (*11*).

Polymethoxyflavones are a group of flavonoids with two or more methoxy groups. There are more than 25 PMFs reported from citrus among which the major occurring are tangeretin (5,6,7,8,4′-pentamethoxyflavone), heptamethoxyflavone (3,5,6,7,8,3’,4’-heptamethoxylflavone), nobiletin (5,6,7,8,3,4’-hexamethoxyflavone), tetramethoxyflavone (5,6,7,4’-tetramethoxyflavanone) and sinensitin (5,6,7,3’,4’-pentamethoxyflavone) (Fig. 1 & 2). While limited literature is available on the evolution of PMFs in citrus, methoxylation of flavone or flavanone aglycones was proposed as a pathway for biosynthesis of PMF’s in oranges (*12*). They occur in leaves, peel and juice but are mainly localized in the peels of the citrus fruits. Studies suggest that the concentration of PMF’s varies based on maturity and species of citrus (*13*, *14*). In plants, PMF’s are considered to be protective against disease causing pathogens (*15–17*). On human health perspective, PMF’s were investigated since early 19th century and implicated in several health benefits such as antiproliferative (*18*), anticancer (*19–22*), anti-inflammatory (*23*), antilipogenic and antimutagenic (*24*) activity. A comprehensive review explaining the multitude health benefits of PMF’s was reported by Li *et al.,* (*25*).

Due to their relevant role in health benefits, PMF’s were isolated from different species and parts of citrus (Table 1). The isolation of PMF’s was achieved by using several extraction and isolation methodologies.

Extraction Methods

Solvent Extraction

Polymethoxyflavones are low polar compounds and can be extracted using non polar solvents such as hexane and polar solvents including water (*18*), ethanol and methanol (*26–28*). Moreover, these compounds were extracted from various parts of citrus such as peel, leaves and cold pressed oil. Raman et al. (*29*), reported extraction of *C. reticulata* peels using non polar hexane solvent followed by treating with 10% sodium hydroxide solution. The mixture was later extracted with diethyl ether, washed with water and subjected to adsorptive separation using cation exchange resin Dowex 50WX2 to yield nobiletin and tangeretin. Chaliha et al. (*30*), reported extraction of *C. jambhiri* peels using petroleum ether solvent in a Soxhlet apparatus for separation of PMF’s. Jayaprakasha et al. (*31*), reported extraction of *C. reticulata* (Blanco Coorg Mandarin) using hexane and chloroform successively in a Soxhlet apparatus. The extracts were subjected to further separation using silica gel column chromatography for isolation of desmethylnobiletin, nobiletin and tangeretin. Miyake (*32*) reported the extraction efficiency of PMF’s using ethanol and aqueous solution of ethanol at various proportions (5%, 25%, 50%, 75% and 100%). Among the evaluated ratios, 75% ethanol in water and 100% ethanol resulted in 100% extraction efficiency. Moreover, extraction of PMF’s was influenced by the temperature of the solvent. Extraction of citrus fruits using hot 25% ethanol in water resulted in higher content of PMF’s in comparison to water, 5% and 25% ethanol aqueous

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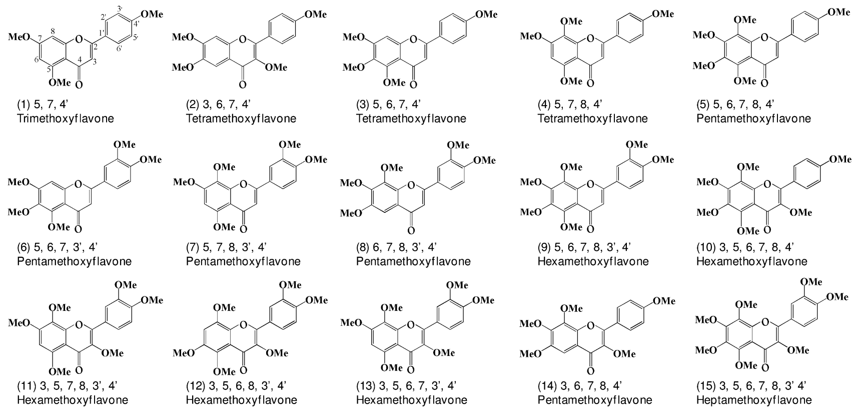
solution under cold and hot water and 5% ethanol in water. Similarly, these compounds were extracted from peels of *C. reticulata* Blanco *cv.* Ponkan by refluxing with 75% ethanol for 3 h (*33*). The ethanol solution was concentrated and further extracted with dichloromethane to yield PMF's rich fraction. Wang et al., (*34*) reported extraction of PMF's from dried peel powder of *C. reticulata* by refluxing with 75% (v/v) ethanol for 10 h. The extract was concentrated and extracted with chloroform to yield mixture of PMF's. Individual PMF's were separated by column chromatography using chloroform:acetone (9:2, v/v). Chen and Montanari (*35*) reported extraction of PMF's from leaves of Dancy tangerine using methanol:chloroform (1:1). The extract was further subjected to separation using combination of flash C_{18} column chromatography and C_{18} preparative HPLC for separating individual PMF's. In a recent report (*36*) from our lab hexane was suggested as a better solvent in comparison to chloroform and methanol solvents for Soxhlet extraction of dried peel powder of Cleopatra mandarin (*C. reshni*). Soxhlet extraction by hexane yielded extract with low occurance of flavonoid glucosides and higher content of PMF's.

Supercritical Fluid Extraction

Apart from citrus peel and leaves, cold pressed oil is a rich source of PMF's. Extraction of these compounds from the precipitate of winterized (storing the oil at -20° C for long duration of time) citrus peel oil was commonly reported (*37–40*). These compounds were also extracted from peel oil extract using super critical fluid extraction (*37*). Recently, the optimum conditions for extraction of nobiletin and tangeretin from *C. depressa* Hayata by supercritical CO_2 was developed by comparing various combinations of pressure and percentage of modifier ethanol solvent. Optimum extraction was achieved by ethanol (85%) as a modifier in supercritical CO_2 maintained at 80 °C and 30 MPa of pressure. Also, the % yield of PMF's by SFE was 107% as compared to conventional solvent extraction yielding 100% (*41*).



(1) 5, 7, 4' Trimethoxyflavone
(2) 3, 6, 7, 4' Tetramethoxyflavone
(3) 5, 6, 7, 4' Tetramethoxyflavone
(4) 5, 7, 8, 4' Tetramethoxyflavone
(5) 5, 6, 7, 8, 4' Pentamethoxyflavone
(6) 5, 6, 7, 3', 4' Pentamethoxyflavone
(7) 5, 7, 8, 3', 4' Pentamethoxyflavone
(8) 6, 7, 8, 3', 4' Pentamethoxyflavone
(9) 5, 6, 7, 8, 3', 4' Hexamethoxyflavone
(10) 3, 5, 6, 7, 8, 4' Hexamethoxyflavone
(11) 3, 5, 7, 8, 3', 4' Hexamethoxyflavone
(12) 3, 5, 6, 8, 3', 4' Hexamethoxyflavone
(13) 3, 5, 6, 7, 3', 4' Hexamethoxyflavone
(14) 3, 6, 7, 8, 4' Pentamethoxyflavone
(15) 3, 5, 6, 7, 8, 3' 4' Heptamethoxyflavone

Figure 1. Structures of polymethoxyflavones isolated from citrus (see Appendix A for larger version of figure).

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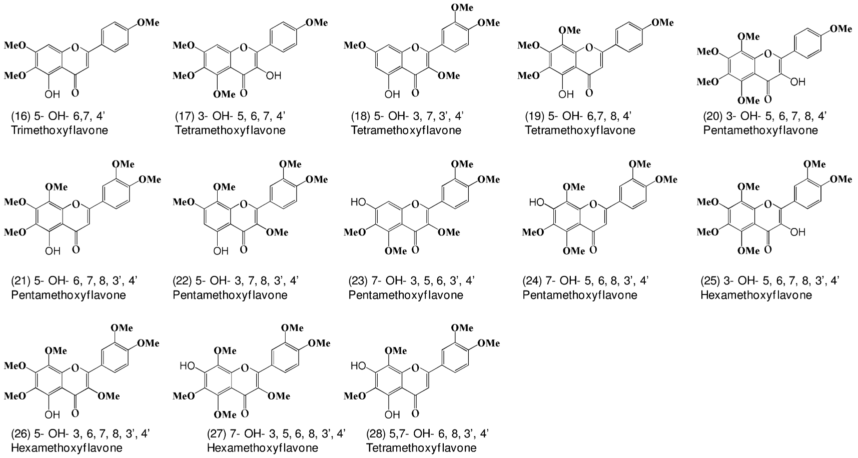

(16) 5- OH- 6,7, 4' Trimethoxyflavone

(17) 3- OH- 5, 6, 7, 4' Tetramethoxyflavone

(18) 5- OH- 3, 7, 3', 4' Tetramethoxyflavone

(19) 5- OH- 6,7, 8, 4' Tetramethoxyflavone

(20) 3- OH- 5, 6, 7, 8, 4' Pentamethoxyflavone

(21) 5- OH- 6, 7, 8, 3', 4' Pentamethoxyflavone

(22) 5- OH- 3, 7, 8, 3', 4' Pentamethoxyflavone

(23) 7- OH- 3, 5, 6, 3', 4' Pentamethoxyflavone

(24) 7- OH- 5, 6, 8, 3', 4' Pentamethoxyflavone

(25) 3- OH- 5, 6, 7, 8, 3', 4' Hexamethoxyflavone

(26) 5- OH- 3, 6, 7, 8, 3', 4' Hexamethoxyflavone

(27) 7- OH- 3, 5, 6, 8, 3', 4' Hexamethoxyflavone

(28) 5,7- OH- 6, 8, 3', 4' Tetramethoxyflavone

Figure 2. Structures of hydroxylated polymethoxyflavones isolated from citrus (see Appendix A for larger version of figure).

Yao et al., (*42*) reported extraction of PMF's from dried peels of *C. sinensis* Osbeck by enzymatic hydrolysis. The dried peel powder (100 g) was extracted exhaustively using 95% ethanol (1,500 mL) and 5% cellulase at 60° C for 2.5 h. The extract was concentrated and treated with diethyl ether (200 mL × 3) and washed with 0.4% sodium hydroxide solution until the extract turned colorless. The clear diethyl ether extract was collected, concentrated, and freeze-dried to obtain crude PMFs (564 mg).

In addition to the traditional extraction methodologies, an advanced technology such as microwave-assisted extraction has also been reported for extraction of PMF's. Dried peels of *C. yuko* Hort. ex Tanaka were refluxed using microwave for 2.5 min to 5 min with methanol yielding 0.12% and 0.10% of tangeretin and nobiletin respectively (*26*).

Separation Methods

Tangeretin was the first PMF isolated from Tangerine (*Citrus nobilis deliciosa*) oil by Nelson (*43*). Nobiletin was isolated from the peels of Chinese mandarin oranges (*C. nobilis*) by Tseng (*44*). Tetramethoxyflavone and heptamethoxyflavone were reported and identified by Swift (*45*) in the neutral fraction of orange peel oil. Sinensitin was, isolated by Born (*46*), and named by Swift (*47*). Although all major PMFs were isolated by the late 60's, extensive isolation was triggered by the implication of these compounds in the several health beneficial properties and consumers' interest in natural products. PMFs were separated using thin layer chromatography (TLC), preparative high performance liquid chromatography (prep-HPLC), supercritical fluid chromatography (SFC), and high speed counter current chromatography (HSCCC).

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Table 1. Polymethoxyflavones isolated from citrus species using different chromatographic methods

Species	*Isolated from*	*Isolation method*	*Isolated PMF's**	*Ref*
C. sinensis L.	Peel oil	FC/prep HPLC	3, 10	(*50*)
C. sinensis	Peel oil	FC/SFC	3, 5, 10, 16	(*37*)
C. sinensis	Peel oil	OC/Flash	16	(*66*)
C. sinensis	Peel oil	Precipitation	10	(*67*)
C. sinensis	Molasses	SEC	3, 5, 7, 10, 14, 16,	(*68*)
C. sinensis Osbeck	Peel extract	OC/TLC/FC	3, 5, 7, 10, 14, 16, 20, 21, 22	(*69*)
C. kinokuni Hort. ex Tanaka	Peel extract	OC/prep-TLC	5, 7, 10, 16, 26	(*70*)
C. nobilis	Juice	OC	5, 10	(*71*)
C. reticulata	Leaves	OC/FC/prep-HPLC	5, 7, 10, 22, 29	(*35*)
C. reticulata Blanco	Peel extract	HSCCC	5, 10, 16, 22	(*54*)
C. reticulata Blanco cv. ponkan	Peel extract	HSCCC	4, 7, 8, 10	(*33*)
C. reticulata (Blanco Coorg mandarin)	Peel extract	OC	5, 10, 22	(*31*)
C. sunki Hort. ex Tanaka	Peel extract	Semi-prep HPLC	1, 4, 5, 7, 8, 10, 20, 22	(*18*)
C. aurantium	Not mentioned	OC	3, 7, 10	(*24*)
C. paradisi	Peel oil	HSCCC	5, 10, 16	(*72*)
C. aurantifolia	Peel oil	HSCCC	5, 10, 16	(*72*)
C. jambhiri Lush	Peel extract	OC	5, 20	(*30*)
C. hassaku	Peel extract	OC/prep-TLC	1, 4, 5, 7, 8, 10, 12, 16	(*49*)
C. yuko Hort	Peel extract	HPLC	5, 10	(*26*)

* The identification and the structure of the isolated PMF's are given in Fig 1 and Fig 2 with the corresponding numerical. Abbreviations: Flash chromatography (FC); Preperative high performance liquid chromatography (Prep HPLC); Supercritical fluid chromatography (SFC); Open column chromatography(OC); Size exclusion chromatography (SEC); Thin layer chromatography (TLC); High speed counter current chromatography (HSCCC).

Preparative Thin Layer Chromatography (prep-TLC)

Among the reported separation methods of PMF's, prep-TLC is the most economical. It is relatively low in cost and does not require sophisticated instrumentation. However, this method is limited by the low amount of sample loaded and yield. Successive separations may be required for obtaining pure PMF's. Del Rio et al., (*48*) reported separation of these compounds from peel oil of various Citrus fruits. Citrus oil was mixed with 2-propanol and distilled water in a decantation funnel and extracted with hexane. The 2-proponol/water phase was concentrated, mixed with water and liquid-liquid extraction was conducted using benzene. The organic phase was separated, concentrated and dehydrated by adding anhydrous sodium sulfate. The extract was placed on a TLC plate containing silica and eluted with benzene:acetone (3:1, v/v). The separated compounds were visualized by their fluorescence and the individual bands were collected and analyzed by HPLC and mass spectrometry.

Machida and Osawa (*49*) reported the isolation of PMF's from the peels of *C. hassaku* using a combination of column chromatography and prep-TLC. Citrus peels were extracted by ethanol under reflux and concentrated. The extract was partitioned between ether and water. The residue was separated on silica gel using benzene-acetone mixture. The components that tested positive in Mg-HCl test were further fractionated by prep-TLC yielding 8 different PMF's.

Preparative-HPLC

Increased interest in investigating the biological activity of PMF's and advancement in chromatographic techniques led to exploring isolation of PMFs using prep-HPLC. Chen at al., (*40*) reported the separation of these compounds from cold pressed Dancy tangerine peel oil solids using prep-HPLC. The procedure involved a combination of normal phase chromatography and C_{18} prep-HPLC. The dried tangerine oil solids were loaded to a open silica gel column and eluted with increasing polarity gradient of benzene/ethyl acetate, ethyl acetate, ethyl acetate/2-propanol and 2-propanol. The fractions with similar PMF's were pooled and purified using C_{18} prep-HPLC with a gradient mobile phase of methanol/water and ethanol/water. The procedure was applied for separation of PMF's from Dancy tangerine leaves leading to the isolation of pure compounds (*35*). However, use of solvents such as benzene for isolation studies should be avoided due to their carcinogenic and mutagenic properties. Li et al., (*50*) reported a gram-scale isolation method of nobiletin using a combination of normal phase flash chromatography and prep-HPLC. The procedure involved initial separation of orange peel extract using silica gel flash column eluted with a gradient solvent system of ethyl acetate and hexanes. The collected fractions containing nobiletin and 5,6,7,4′-tetramethoxyflavone were concentrated and further separated on a Regis chiral column connected to a prep-HPLC. The solvent system consisted of 35% ethanol and 65% hexanes with a flow rate set at 85 mL/min resulting in isolation of gram amounts of nobiletin and 5,6,7,4′-tetramethoxyflavone. Similar procedure was further applied for isolation of other PMF's from cold-pressed orange peel oil (*51*).

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Supercritical Fluid Chromatography

This method is one of the ideal methods for separation of PMF's. This method involves use of pressure and temperature combinations maintained at critical point of the mobile phase used. Moreover, the absence of permanent adsorptive loss of sample on to the stationary phase which is commonly noticed in column chromatography makes this method advantageous. Among the various mobile phases used for SFC, CO_2 along with methanol seems to be ideal for separation of PMF's. This method was initially used for analyzing the authenticity of citrus oils by quantification of PMF's (*52*). The separation of PMF's was conducted using CO_2 as mobile phase and methanol as a polar modifier. In an another report hydroxy- and methoxy-flavones were separated by supercritical CO_2 chromatography on capillary columns using flame ionization and Fourier transform infrared (FT-IR) spectroscopy detection (*53*). Recently, a large scale isolation method of four PMF's such as nobiletin, tangeretin, 3,5,6,7,8,3′,4′-heptamethoxyflavone and 5,6,7,4′-tetramethoxyflavone was reported using a combination of normal phase flash column separation and SFC separation (*37*). The raw material used for the separation was crude sweet orange peel extract. The extract was separated on a silica gel flash column using a gradient mobile phase. Individual fractions were analyzed by LC–ESI-MS and TLC and grouped into 6 groups. The groups that had high concentration of PMF's were subjected to SFC separation using mobile phase of CO_2 and methanol. The separated peaks were collected as individual fractions to obtain pure PMF's.

High-Speed Counter Current Chromatography

This is a chromatography technique in which liquid–liquid partition is used as a strategy for separations and unlike other chromatographic techniques does not use any solid support matrix. Due to the characteristic absence of solid support matrix there is no loss of samples by adsorption. This method was first reported as efficient method for the preparative isolation and purification of polymethoxylated flavones from Tangereine peel extracts (*54*). Tangerine peels were extracted by light petroleum, concentrated and frozen. The sediment was dried and injected to the HSCCC in 15 mL sample injections. The separations were conducted using a two-phase solvent system composed of *n*-hexane, ethyl acetate, methanol and water (1:0.8:1:1) (v/v). The effluent was monitored with a UV detector at 254 nm and peak fractions were collected according to the elution profile. Similar peaks were pooled and four PMF's nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, tangeretin and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone were isolated in milligram quantity.

Flash Chromatography

FC also called as medium pressure liquid chromatography which is a faster technique of column chromatography. The regulated application of medium pressure enables separation of compounds using large sample volumes, thereby yielding high quantity of pure compounds. Recent technological advances have also enabled conducting separations with online detection and robotic fraction collectors. These advancements have enabled in development of large scale separation of PMF's. Dried peels of Cleopatra mandarin and Marrs sweet orange (*C. sinensis L.* Osbeck) fruits were powdered and extracted using a non polar solvent in a Soxhlet. The extract was concentrated, impregnated with silica gel and subjected to separation using flash chromatography. A gradient solvent system was used for separation and the eluent was monitored at wavelengths of 254 nm & 340 nm. Individual peaks were collected in fractions and pooled after analyzing by HPLC. The isolated compounds were identified as sinensitin, tetramethoxyflavone, nobiletin, and tangeretin using NMR and mass spectrometry (*36*, *55*).

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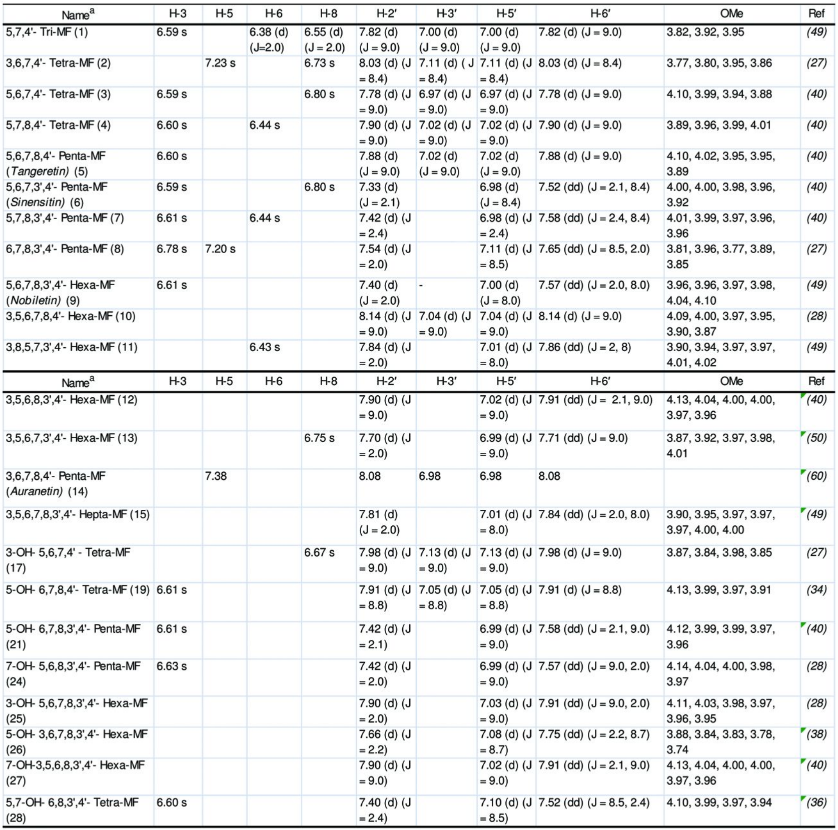
Identification and Structure Elucidation

Identification of PMF's is challenging due to their close similarity in structures and as well as molecular weight. This necessitates use of proper tools and techniques to determine their exact structure. Until late 70's, infrared spectroscopic studies coupled with degradation and synthetic studies were commonly used for elucidating the structure of PMF's. Although degradation and synthetic studies were used for structural analysis, IR analysis played an important role in confirmation of the structure of PMF's. One such example can be given as the ambiguity in the flavonol constitution of a compound synthesized by Goldsworth and Robinson (*56*). The compound was considered identical to tangeretin as suggested by degradation and synthetic studies which was later proved to be different from that of the actual structure of tangeretin given as 5,6,7,8,4′-pentamethoxyflavone confirmed by the IR spectroscopy (*57*). These early investigations on the structure of isolated PMF's were comprehensively reviewed by Sarin and Sheshadri (*58*). In modern era of chemistry, nuclear magnetic resonance (NMR) (*28*, *31*, *34–36*, *40*, *49*, *51*, *59*, *60*) is used for accurate structure elucidation. The importance of ^{13}C NMR and its application for identification of flavonoids was reviewed by Agrawal (*61*). The ^{1}H NMR and ^{13}C NMR of the reported PMF's are summarized in Table 2 and 3 respectively. However, this method is limited by the requirement of large quantity of purified compounds. Other spectral analysis techniques used for identification of PMF's were gas chromatography- mass spectrometry (GC-MS) (*62*, *63*), and LC-MS (*64*, *65*). These techniques provide valuable information in regards to the compounds molecular weight along with the fragment ions. The advantage of minimal sample requirement for identification provides a valuable tool for structure elucidation. Raman et al., (*29*) reported identification of nobiletin and tangeretin using mass spectrometry in negative electronspray ionization (ESI) mode. The structures were further elucidated by collisional activated dissociation (CAD) to generate fragmentation patterns of the deprotonated flavones. Wang and Zhang (*64*) reported positive electronspray ionization tandem mass spectrometry of PMF's. Although NMR and MS studies provide structure information of individual isolated compounds, these methods are limited in application for identification of components in crude extracts. Recently, Weber et al., (*38*) reported the LC-NMR method for identification PMF's present in residues from molecular distillation of cold pressed peel oils of *C. sinensis*. The individual PMF's were initially separated using HPLC followed by NMR analysis conducted in the stop-flow mode.

Table 2. ^{1}H NMR of polymethoxyflavones isolated from citrus species (see Appendix A for larger version of table)



Name[a]	H-3	H-5	H-6	H-8	H-2′	H-3′	H-5′	H-6′	OMe	Ref
5,7,4'- Tri-MF (1)	6.59 s		6.38 (d) (J=2.0)	6.55 (d) (J = 2.0)	7.82 (d) (J = 9.0)	7.00 (d) (J = 9.0)	7.00 (d) (J = 9.0)	7.82 (d) (J = 9.0)	3.82, 3.92, 3.95	*(49)*
3,6,7,4'- Tetra-MF (2)		7.23 s		6.73 s	8.03 (d) (J = 8.4)	7.11 (d) (J = 8.4)	7.11 (d) (J = 8.4)	8.03 (d) (J = 8.4)	3.77, 3.80, 3.95, 3.86	*(27)*
5,6,7,4'- Tetra-MF (3)	6.59 s			6.80 s	7.78 (d) (J = 9.0)	6.97 (d) (J = 9.0)	6.97 (d) (J = 9.0)	7.78 (d) (J = 9.0)	4.10, 3.99, 3.94, 3.88	*(40)*
5,7,8,4'- Tetra-MF (4)	6.60 s		6.44 s		7.90 (d) (J = 9.0)	7.02 (d) (J = 9.0)	7.02 (d) (J = 9.0)	7.90 (d) (J = 9.0)	3.89, 3.96, 3.99, 4.01	*(40)*
5,6,7,8,4'- Penta-MF (*Tangeretin*) (5)	6.60 s				7.88 (d) (J = 9.0)	7.02 (d) (J = 9.0)	7.02 (d) (J = 9.0)	7.88 (d) (J = 9.0)	4.10, 4.02, 3.95, 3.95, 3.89	*(40)*
5,6,7,3',4'- Penta-MF (*Sinensitin*) (6)	6.59 s			6.80 s	7.33 (d) (J = 2.1)		6.98 (d) (J = 8.4)	7.52 (dd) (J = 2.1, 8.4)	4.00, 4.00, 3.98, 3.96, 3.92	*(40)*
5,7,8,3',4'- Penta-MF (7)	6.61 s		6.44 s		7.42 (d) (J = 2.4)		6.98 (d) (J = 2.4)	7.58 (dd) (J = 2.4, 8.4)	4.01, 3.99, 3.97, 3.96, 3.96	*(40)*
6,7,8,3',4'- Penta-MF (8)	6.78 s	7.20 s			7.54 (d) (J = 2.0)		7.11 (d) (J = 8.5)	7.65 (dd) (J = 8.5, 2.0)	3.81, 3.96, 3.77, 3.89, 3.85	*(27)*
5,6,7,8,3',4'- Hexa-MF (*Nobiletin*) (9)	6.61 s				7.40 (d) (J = 2.0)	-	7.00 (d) (J = 8.0)	7.57 (dd) (J = 2.0, 8.0)	3.96, 3.96, 3.97, 3.98, 4.04, 4.10	*(49)*
3,5,6,7,8,4'- Hexa-MF (10)					8.14 (d) (J = 9.0)	7.04 (d) (J = 9.0)	7.04 (d) (J = 9.0)	8.14 (d) (J = 9.0)	4.09, 4.00, 3.97, 3.95, 3.90, 3.87	*(28)*
3,8,5,7,3',4'- Hexa-MF (11)			6.43 s		7.84 (d) (J = 2.0)		7.01 (d) (J = 8.0)	7.86 (dd) (J = 2, 8)	3.90, 3.94, 3.97, 3.97, 4.01, 4.02	*(49)*
3,5,6,8,3',4'- Hexa-MF (12)					7.90 (d) (J = 9.0)		7.02 (d) (J = 9.0)	7.91 (dd) (J = 2.1, 9.0)	4.13, 4.04, 4.00, 4.00, 3.97, 3.96	*(40)*
3,5,6,7,3',4'- Hexa-MF (13)				6.75 s	7.70 (d) (J = 2.0)		6.99 (d) (J = 9.0)	7.71 (dd) (J = 9.0)	3.87, 3.92, 3.97, 3.98, 4.01	*(50)*
3,6,7,8,4'- Penta-MF (*Auranetin*) (14)		7.38			8.08	6.98	6.98	8.08		*(60)*
3,5,6,7,8,3',4'- Hepta-MF (15)					7.81 (d) (J = 2.0)		7.01 (d) (J = 8.0)	7.84 (dd) (J = 2.0, 8.0)	3.90, 3.95, 3.97, 3.97, 3.97, 4.00, 4.00	*(49)*
3-OH- 5,6,7,4' - Tetra-MF (17)				6.67 s	7.98 (d) (J = 9.0)	7.13 (d) (J = 9.0)	7.13 (d) (J = 9.0)	7.98 (d) (J = 9.0)	3.87, 3.84, 3.98, 3.85	*(27)*
5-OH- 6,7,8,4'- Tetra-MF (19)	6.61 s				7.91 (d) (J = 8.8)	7.05 (d) (J = 8.8)	7.05 (d) (J = 8.8)	7.91 (d) (J = 8.8)	4.13, 3.99, 3.97, 3.91	*(34)*
5-OH- 6,7,8,3',4'- Penta-MF (21)	6.61 s				7.42 (d) (J = 2.1)		6.99 (d) (J = 9.0)	7.58 (dd) (J = 2.1, 9.0)	4.12, 3.99, 3.99, 3.97, 3.96	*(40)*
7-OH- 5,6,8,3',4'- Penta-MF (24)	6.63 s				7.42 (d) (J = 2.0)		6.99 (d) (J = 9.0)	7.57 (dd) (J = 9.0, 2.0)	4.14, 4.04, 4.00, 3.98, 3.97	*(28)*
3-OH- 5,6,7,8,3',4'- Hexa-MF (25)					7.90 (d) (J = 2.0)		7.03 (d) (J = 9.0)	7.91 (dd) (J = 9.0, 2.0)	4.11, 4.03, 3.98, 3.97, 3.96, 3.95	*(28)*
5-OH- 3,6,7,8,3',4'- Hexa-MF (26)					7.66 (d) (J = 2.2)		7.08 (d) (J = 8.7)	7.75 (dd) (J = 2.2, 8.7)	3.88, 3.84, 3.83, 3.78, 3.74	*(38)*
7-OH-3,5,6,8,3',4'- Hexa-MF (27)					7.90 (d) (J = 9.0)		7.02 (d) (J = 9.0)	7.91 (dd) (J = 2.1, 9.0)	4.13, 4.04, 4.00, 4.00, 3.97, 3.96	*(40)*
5,7-OH- 6,8,3',4'- Tetra-MF (28)	6.60 s				7.40 (d) (J = 2.4)		7.10 (d) (J = 8.5)	7.52 (dd) (J = 8.5, 2.4)	4.10, 3.99, 3.97, 3.94	*(36)*

[a] The structures are given in Fig 1 and Fig 2 which correspond to the numerical given in brackets.

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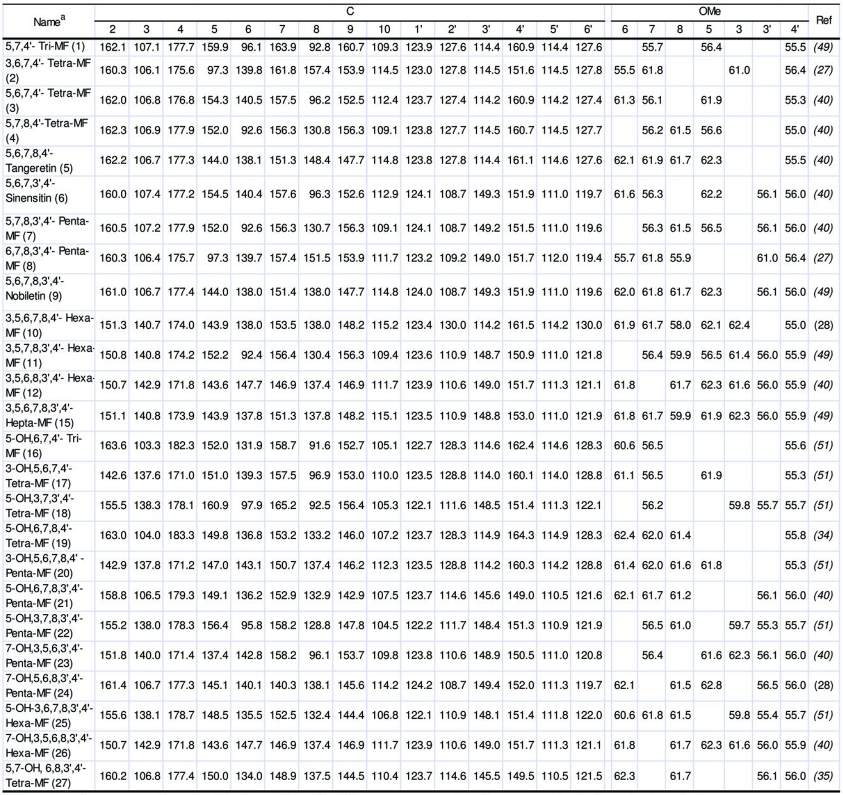
Table 3. ^{13}C NMR of polymethoxyflavones isolated from citrus species (see Appendix A for larger version of table)



Name[a]	C															OMe							Ref
	2	3	4	5	6	7	8	9	10	1'	2'	3'	4'	5'	6'	6	7	8	5	3	3'	4'	
5,7,4'- Tri-MF (1)	162.1	107.1	177.7	159.9	96.1	163.9	92.8	160.7	109.3	123.9	127.6	114.4	160.9	114.4	127.6		55.7		56.4			55.5	*(49)*
3,6,7,4'- Tetra-MF (2)	160.3	106.1	175.6	97.3	139.8	161.8	157.4	153.9	114.5	123.0	127.8	114.5	151.6	114.5	127.8	55.5	61.8			61.0		56.4	*(27)*
5,6,7,4'- Tetra-MF (3)	162.0	106.8	176.8	154.3	140.5	157.5	96.2	152.5	112.4	123.7	127.4	114.2	160.9	114.2	127.4	61.3	56.1		61.9			55.3	*(40)*
5,7,8,4'-Tetra-MF (4)	162.3	106.9	177.9	152.0	92.6	156.3	130.8	156.3	109.1	123.8	127.7	114.5	160.7	114.5	127.7		56.2	61.5	56.6			55.0	*(40)*
5,6,7,8,4'- Tangeretin (5)	162.2	106.7	177.3	144.0	138.1	151.3	148.4	147.7	114.8	123.8	127.8	114.4	161.1	114.6	127.6	62.1	61.9	61.7	62.3			55.5	*(40)*
5,6,7,3',4'- Sinensitin (6)	160.0	107.4	177.2	154.5	140.4	157.6	96.3	152.6	112.9	124.1	108.7	149.3	151.9	111.0	119.7	61.6	56.3		62.2		56.1	56.0	*(40)*
5,7,8,3',4'- Penta-MF (7)	160.5	107.2	177.9	152.0	92.6	156.3	130.7	156.3	109.1	124.1	108.7	149.2	151.5	111.0	119.6		56.3	61.5	56.5		56.1	56.0	*(40)*
6,7,8,3',4'- Penta-MF (8)	160.3	106.4	175.7	97.3	139.7	157.4	151.5	153.9	111.7	123.2	109.2	149.0	151.7	112.0	119.4	55.7	61.8	55.9			61.0	56.4	*(27)*
5,6,7,8,3',4'- Nobiletin (9)	161.0	106.7	177.4	144.0	138.0	151.4	138.0	147.7	114.8	124.0	108.7	149.3	151.9	111.0	119.6	62.0	61.8	61.7	62.3		56.1	56.0	*(49)*
3,5,6,7,8,4'- Hexa-MF (10)	151.3	140.7	174.0	143.9	138.0	153.5	138.0	148.2	115.2	123.4	130.0	114.2	161.5	114.2	130.0	61.9	61.7	58.0	62.1	62.4		55.0	(28)
3,5,7,8,3',4'- Hexa-MF (11)	150.8	140.8	174.2	152.2	92.4	156.4	130.4	156.3	109.4	123.6	110.9	148.7	150.9	111.0	121.8		56.4	59.9	56.5	61.4	56.0	55.9	*(49)*
3,5,6,8,3',4'- Hexa-MF (12)	150.7	142.9	171.8	143.6	147.7	146.9	137.4	146.9	111.7	123.9	110.6	149.0	151.7	111.3	121.1	61.8		61.7	62.3	61.6	56.0	55.9	*(40)*
3,5,6,7,8,3',4'- Hepta-MF (15)	151.1	140.8	173.9	143.9	137.8	151.3	137.8	148.2	115.1	123.5	110.9	148.8	153.0	111.0	121.9	61.8	61.7	59.9	61.9	62.3	56.0	55.9	*(49)*
5-OH,6,7,4'- Tri-MF (16)	163.6	103.3	182.3	152.0	131.9	158.7	91.6	152.7	105.1	122.7	128.3	114.6	162.4	114.6	128.3	60.6	56.5					55.6	*(51)*
3-OH,5,6,7,4'- Tetra-MF (17)	142.6	137.6	171.0	151.0	139.3	157.5	96.9	153.0	110.0	123.5	128.8	114.0	160.1	114.0	128.8	61.1	56.5		61.9			55.3	*(51)*
5-OH,3,7,3',4'- Tetra-MF (18)	155.5	138.3	178.1	160.9	97.9	165.2	92.5	156.4	105.3	122.1	111.6	148.5	151.4	111.3	122.1		56.2			59.8	55.7	55.7	*(51)*
5-OH,6,7,8,4'- Tetra-MF (19)	163.0	104.0	183.3	149.8	136.8	153.2	133.2	146.0	107.2	123.7	128.3	114.9	164.3	114.9	128.3	62.4	62.0	61.4				55.8	*(34)*
3-OH,5,6,7,8,4' - Penta-MF (20)	142.9	137.8	171.2	147.0	143.1	150.7	137.4	146.2	112.3	123.5	128.8	114.2	160.3	114.2	128.8	61.4	62.0	61.6	61.8			55.3	*(51)*
5-OH,6,7,8,3',4'- Penta-MF (21)	158.8	106.5	179.3	149.1	136.2	152.9	132.9	142.9	107.5	123.7	114.6	145.6	149.0	110.5	121.6	62.1	61.7	61.2			56.1	56.0	*(40)*
5-OH,3,7,8,3',4'- Penta-MF (22)	155.2	138.0	178.3	156.4	95.8	158.2	128.8	147.8	104.5	122.2	111.7	148.4	151.3	110.9	121.9		56.5	61.0		59.7	55.3	55.7	*(51)*
7-OH,3,5,6,3',4'- Penta-MF (23)	151.8	140.0	171.4	137.4	142.8	158.2	96.1	153.7	109.8	123.8	110.6	148.9	150.5	111.0	120.8		56.4		61.6	62.3	56.1	56.0	*(40)*
7-OH,5,6,8,3',4'- Penta-MF (24)	161.4	106.7	177.3	145.1	140.1	140.3	138.1	145.6	114.2	124.2	108.7	149.4	152.0	111.3	119.7	62.1		61.5	62.8		56.5	56.0	(28)
5-OH-3,6,7,8,3',4'- Hexa-MF (25)	155.6	138.1	178.7	148.5	135.5	152.5	132.4	144.4	106.8	122.1	110.9	148.1	151.4	111.8	122.0	60.6	61.8	61.5		59.8	55.4	55.7	*(51)*
7-OH,3,5,6,8,3',4'- Hexa-MF (26)	150.7	142.9	171.8	143.6	147.7	146.9	137.4	146.9	111.7	123.9	110.6	149.0	151.7	111.3	121.1	61.8		61.7	62.3	61.6	56.0	55.9	*(40)*
5,7-OH, 6,8,3',4'- Tetra-MF (27)	160.2	106.8	177.4	150.0	134.0	148.9	137.5	144.5	110.4	123.7	114.6	145.5	149.5	110.5	121.5	62.3		61.7			56.1	56.0	*(35)*

[a] The structures are given in Fig 1 and Fig 2 which correspond to the numerical given in brackets.

Summary

Several extraction methods, isolation techniques and identification strategies were reported for PMF's. Overall review suggests that majority of the research on isolation of PMF's is limited to only few species of citrus. Future efforts should be focused on exploring PMF's in citrus species that have not been studied earlier. Moreover emphasis should be made to develop efficient large scale isolation methods of PMF's using food grade solvents in order to benefit further *in vivo* studies on disease prevention.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Chapter 2

Flavonoids and Furocoumarins in Bergamot, Myrtle-Leaved Orange, and Sour Orange Juices: Distribution and Properties

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Citrus juices are among the richest dietary sources of health-promoting compounds. Beyond the mainstream species (such as orange, grape or lemon), a wide number of other *Citrus* species are grown around the world, but their composition and properties have not been fully studied. The flavonoid and furocoumarin distribution in juices from *C. aurantium*, *C. myrtifolia* and *C. bergamia* will be described and discussed in a critical fashion, along with the ability of selected flavonoids to activate apoptotic signal cascade and to inhibit hepatoma cells proliferation.

Introduction

The beneficial influence of *Citrus* juices consumption on human health has been known for centuries, well before researchers started to unravel the complexity of such food matrices in the attempt to gain a deeper understanding on the correlation between diet and health benefits.

Citrus juices are among the richest dietary sources of health-promoting compounds (*1*, *2*), but there is still a lot to be done before having a clear and detailed picture of the exact composition of such natural treasure troves. They have been shown to contain many different classes of bioactive compounds such as, to name a few, vitamins, flavonoids, furocoumarins, limonoids or phytosterols. As a matter of fact, they have been, and still are, the object of a large number of

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investigations, both from the analytical (i.e. identification and quantification), and the biological (*in vitro* and *in vivo* activity, epidemiological studies) point of view.

Flavonoids, in particular, occur in *Citrus* juices in significant amount (*3–5*), and they have attracted particular attention as a result of their remarkable antioxidant activity, which is responsible of a wide spectrum of beneficial properties (*6*). Noticeable examples are the studies reported on their antiviral (*7*), antimicrobial (*8*), and anti-inflammatory (*9*), antiulcer (*10*) and antiallergenic properties (*11*), and on their ability to inhibit human platelet aggregation (*12*). Furthermore, there is strong leading evidence that they provide protection against some cardiovascular disorders (*13*) and cancerous processes (*14*), even though their *in vivo* mechanism of action has not been fully understood (*15*, *16*).

To date, more than 60 flavonoids have been identified in the juice fraction of *Citrus* fruits (*17*). They mostly belong to the flavanone and flavone subclasses, the former representing usually the most abundant components, the latter being present in wider variety. Along to these compounds, in some cases smaller amounts of flavonols and isoflavones have also been found. Anthocyanidines have been detected in pigmented juices (*18*).

Flavonoids, actually, are present in all the parts of the fruit, namely the flavedo, albedo, juice vesicles and pith. However, where the most lipophilic derivatives (polymethoxyflavones, aglycones) tend to accumulate in the essential oil fraction of the rind (*19*), in the juice the polyphenolic compounds are usually found as their glycosylated forms. Interestingly, all the flavonoid glycosides identified so far in *Citrus* juices bear glucose units or disaccharide moieties composed of rhamnose and glucose, connected either in a 1→2 or a 1→6 fashion (α-(1→2)-L-rhamnopyranosyl-β-D-glucopyranose, neohesperidose; α-(1→6)-L-rhamnopyranosyl-β-D-glucopyranose, rutinose). In flavanones and flavones, neohesperidose and rutinose are generally found linked to the 7-*O*-position of the aglycone, with the noticeable exception of the flavonol, quercetin, which bears a rutinose substituent attached to the 3-*O*-position of the C-ring. Glucose is sometimes found in the 4′-*O*-position (i.e. on the B ring). Several studies (*20*) described also a series of flavones bearing sugar moieties (again glucose, neohesperidose or rutinose) attached to the aglycone via *C*-glycosidic rather than *O*-glycosidic bonds as in the cases described above.

All these compounds possess distribution patterns that are unique to each species. They can be effectively regarded as chromatographic fingerprints (*21*), allowing for a direct assessment of quality and/or adulteration of a given juice, and they can also be employed for the identification of taxonomical relation between different species. Lemons, for instance, are characterized by an abundance of eriocitrin, hesperidin, and diosmin (*22*, *23*), whereas hesperidin, narirutin, and didymin are typical of sweet oranges (*19*, *24*); naringin, narirutin and, to a lesser extent, hesperidin and neohesperidin, are found in grapefruit species (*21*). As far as furocoumarins are concerned, the derivatives identified so far mostly belong to the psoralen (i.e. linear furocoumarins) class, generally bearing an alkoxy substituent in position 5.

However, beyond the mainstream juices such as the mentioned orange, grape or lemon (which have been the subject of countless investigations), a wide number of other *Citrus* species are grown in significant amount and used for different

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applications, but their flavonoid distributions have not been fully investigated. As a part of our research in this area, over the past few years we have optimized a procedure, based on HPLC-DAD-ESI-MS-MS, which permits to analyze directly the crude juice obtained from *Citrus* fruits, and allows for the identification and quantification, in a single chromatographic run, of a large number of flavonoid glycosides and furocoumarins. In the present paper, we report, in a critical fashion, the results obtained from our investigations on three closely related taxons, *Citrus aurantium* Swingle (sour orange), *Citrus* × *myrtifolia* Raf. (myrtle-leaved orange or chinotto), a mutation of sour orange, and *Citrus bergamia* Risso (bergamot), which is regarded as a cross between sour orange and citron (*C. medica*, (*25*)), even though other studies describe it as a cross with *C. limetta* (*26*). An updated description of their flavonoid and furocoumarin distributions is presented, and the *in vitro* antioxidant and antiproliferative activity of some selected flavonoids characteristic of *Citrus* juices are discussed.

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Results and Discussion

Identification and Quantification Procedures

Flavonoids and furocoumarins analysis over the years has been performed by several different techniques (*17*); however, the methods that provided the best results rely all on the use of liquid chromatography coupled to UV or MS detection. In our case, the juices examined were directly subjected to HPLC-DAD coupled with ESI-MS-MS (*27*). This method presents several advantages, the first one being the possibility to proceed without any preliminary extraction. In fact, juices were diluted with DMF (1:1 v/v), centrifuged, filtered and directly injected for analysis on a reverse-phase C-18 column, using an acetonitrile/water gradient as the mobile phase. Noticeably, under these conditions di-*C*-glucosyl flavones are eluted first, followed by tri-*O*-glycosyl flavones and flavanones, mono-*C*-glucosyl flavones, di-*O*-glycosyl flavones and flavanones, flavanones bearing carboxylic acid residues, and finally furocoumarins.

Along with retention times, diode array detection allows for a convenient preliminary identification of the aglycone skeleton. In fact, flavanones and flavones present significant differences in the UV-Vis absorption spectra: they both possess a strong band at λ = *ca.* 270–280 nm (conventionally known as band II), which is generally attributed to the dihydroxybenzoyl portion of the molecule (i.e. the conjugated π-system ring A/carbonyl group). Conversely, only flavones possess a strong absorption band at λ = *ca.* 320–330 nm (band I, attributed to the styrene-type π-system represented by ring B and the 2,3 double bond in ring C, which is absent in flavanones). Therefore, comparison of the chromatograms, recorded simultaneously at 278 and 325 nm, allowed for a facile structural assignment of analytes belonging either to the flavone class (peaks present in the chromatograms recorded at both wavelengths) or to the flavanone class (present only in the chromatograms recorded at 278 nm). In addition, detection at λ = 310 nm facilitated the identification of furocoumarin nuclei, as they possess an absorption maximum around 310-315 nm.

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A second structural sorting could be carried out by inspection of the chromatograms obtained from samples of juices that had been subjected to acidic hydrolysis. This procedure permitted to discriminate between flavonoid *O*-glycosides and *C*-glycosides, given that *O*-glycosidic linkages are hydrolyzed in aqueous HCl (1.2 M, 90 °C, 20 h), whereas *C*-glycosidic bonds are resistant in under these conditions.

MS and MS^2 spectra, recorded for each peak both in positive and negative ion mode, have proven to be powerful tools for the final structural elucidation of these compounds. Once a given analyte has been temporarily identified as an *O*- or *C*-glycosyl flavone/flavanone, MS^2 provides information on both the nature of the aglycone (henceforth referred to as 'A') (*28*), and the nature, number, position and type of the glycosidic linkage of the saccharide units (*29–32*). In fact, fragmentation patterns differ sensibly, depending even on very minor structural variation of the analyte. *O*-/*C*-glycosidic linkage discrimination is confirmed, in the former case, by the neutral loss of the entire sugar moiety whereas, in the latter, by the telltale appearance in the negative ion mode spectrum of typical ions ($[M–H–18]^-$, $[M–H–90]^-$, $[M–H–120]^-$) derived from the fragmentation of the pyranosidic ring itself, along with $[A+41]^-$, $[A+71]^-$ for mono-*C*-glucosides, or $[A+83]^-$, $[A+113]^-$ for di-*C*-glucosides (*32*). The position of the interglycosidic bond in rutinose and neohesperidose (i.e. 1→6 vs. 1→2) can be easily revealed by the fragmentation sequence of the disaccharide moiety in negative ion mode spectra focused on $[M–H]^-$. In the case of rutinose, the progressive loss of entire neutral monosaccharide units is observed ($[M–H–Rha]^-$, $[M–H–Rha–Glu]^-$). Conversely, when neohesperidose is present, a peak for the ion $[M–H–120]^-$ is observed, resulting from the fragmentation of the glucose unit, followed by the loss of rhamnose ($[M–H–120–Rha]^-$) (*29*). Further details can be found in recent articles that have extensively covered the subject (*29–32*).

As far as the quantification of analytes is concerned, a selected reaction monitoring (SRM) procedure was employed (*33*). It was found to be far superior to UV detection, giving access to very precise determination of all the identified flavonoids and furocoumarins, even in the presence of partially overlapping peaks.

Analysis of Juices

The juices of sour orange (*Citrus aurantium*), myrtle-leaved orange (*Citrus* × *myrtifolia*) and bergamot (*Citrus bergamia* Risso) were prepared by hand-squeezing fruits from which the peel had been preliminary removed, in order to avoid contamination of the juices by polymethoxyflavones and other components from the essential oil fraction of the peel (*19*). The compounds identified and quantified in these food matrices (Figures 1–3) are collectively reported in Tables I–III.

Sour orange (also known as bitter orange) has been attracting attention in recent times, since it has been widely employed in the production of food supplements for appetite control. It is known to contain a powerful alkaloid, synephrine (*34*), as well as significant amounts of bioactive limonoids and phytosterols (*35*). Sour orange juice (*36*) was found to be characterized by the abundance of the flavanones, naringin **10** and neohesperidin **11**, followed closely by another flavanone, neoeriocitrin **7** (23.37 ± 1.48, 18.9 ± 1.18 and 9.03 ± 0.84 mg/L, respectively, see Figure 1). Interestingly, they are all 7-*O*-neohesperidosyl derivatives. These compounds were already known to be the preeminent components of sour orange juice (*37*). There are, however, several other components, such as the flavanones melitidin **12** and brutieridin **13** (3.74 ± 0.47 and 6.49 ± 0.77 mg/L, respectively), which are naringin and hesperidin derivatives bearing at the 6-position of their glucose units a 3-hydroxy-3-methyl-glutaryl (HMG) substituent. These two compounds have been indicated as potentially relevant in the treatment of hypercholesterolemia (*38*). Other relevant components are the *C*-glucosyl flavones. Vicenin-2 **2**, a 6,8-di-*C*-glucosyl apigenin derivative, is the most abundant (1.54 ± 0.21 mg/L) but, as it has been demonstrated for other *Citrus* species (*39*), it is accompanied by other minor components belonging to the di-*C*-glucosyl flavone family (**1**, **3**, **4**). Rhoifolin **9** and rhoifolin 4′-*O*-glucoside **4** were also found. Interestingly, rutinosides (which are typical of sweet orange, lemon and tangerines, for instance (*17*)) are scarcely present in sour orange juice, the only examples being small amounts of eriocitrin **6**, narirutin **8** and traces of 4′-*O*-glucosylated narirutin **4**. The only furocoumarin derivative found was epoxybergamottin **14**. This came rather as a surprise, since most of the time furocoumarins are found as components of the essential oil fraction of the peel, even though in grapefruit they are known to be present (*17*) and responsible for the so-called grapefruit effect (the interaction of grapefruit juice with some drugs (*40*)).

Myrtle-leaved orange (or chinotto) is mostly grown as an ornamental plant, but it is also the key ingredient for the production of soft drinks and liqueurs. When myrtle-leaved orange juice was analyzed, it was found to be remarkably similar to sour orange juice, even though its total flavonoid content was almost four times lower, i.e. 18.3 ± 1.6 mg /L (*41*) versus 65.6 ± 1.4 mg /L (*36*).

Naringin **10**, neohesperidin **11**, followed by neoeriocitrin **7** (in this order, 6.08 ± 0.16, 5.76 ± 0.17 and 3.08 ± 0.11 mg/L, respectively), were again the most characteristic components (Figure 2). The HMG-neohesperidosyl flavanones brutieridin **13** and melitidin (**12**, hereby reported for the first time) were found to occur in significant amount (2.19 ± 0.18 and 1.91 ± 0.17 mg/L, respectively). Furthermore, more recent investigations allowed us to present, along to the mentioned melitidin **12**, an additional three new components which had not been reported previously. Trace amounts (< 1 mg/L) of lucenin-2 **1**, and of the tri-*O*-glycosides, rhoifolin 4′-*O*-glucoside **4** and narirutin 4′-*O*-glucoside **5**, were also found in myrtle-leaved orange for the first time. Also in this case, the only rutinosides found were eriocitrin **6**, narirutin 4′-*O*-glucoside **4** and traces of narirutin **8**. Interestingly, both in *C. aurantium* and *C. myrtifolia* all the flavanones identified are hesperidin, naringenin and eriodictyol derivatives, whereas the flavones, with the exception of a luteolin derivative (**1**), all derive from apigenin and diosmetin. As for the furocoumarins, bergapten **15** and epoxybergamottin **14** were found in small amount (< 1 mg/L).

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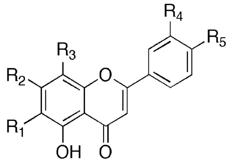
Table I. Flavone-*C*-glucosides (1-4, 8, 10 and 11) and flavone-*O*-glycosides (5, 6, 12, 14 and 15)



	R_1	R_2	R_3	R_4	R_5	Structure assignment
1	Glu	OH	Glu	OH	OH	Luteolin 6,8-di-*C*-glucoside (Lucenin-2)
2	Glu	OH	Glu	H	OH	Apigenin 6,8-di-*C*-glucoside (Vicenin-2)
3	Glu	OH	Glu	OH	OMe	Diosmetin 6,8-di-*C*-glucoside (Lucenin-2 4′-methyl ether)
4	H	*O*-Nh[a]	H	H	*O*-Glu	Apigenin 7-*O*-neohesperidoside-4′-*O*-glucoside (Rhoifolin 4′-glucoside)
9	H	*O*-Nh[a]	H	H	OH	Apigenin 7-*O*- neohesperidoside (Rhoifolin)
16	Glu	OH	Glu	OMe	OH	Chrysoeriol 6,8-di-*C*-glucoside (Stellarin-2)
17	H	*O*-Nh[a]	H	OMe	OH	Chrysoeriol 7-*O*-neohesperidoside-4′-*O*-glucoside
18	Glu	OH	H	H	OH	Apigenin 6-*C*-glucoside (Isovitexin)
19	H	OH	Glu	OMe	OH	Chrysoeriol 8-*C*-glucoside (Scoparin)
20	H	OH	Glu	OH	OMe	Diosmetin 8-*C*-glucoside (Orientin 4′-methyl ether)
21	H	*O*-Nh[a]	H	OMe	OH	Chrysoeriol 7-*O*-neohesperidoside
22	H	*O*-Nh[a]	H	OH	OMe	Diosmetin 7-*O*-neohesperidoside (Neodiosmin)

[a] *O*-Neohesperidose.

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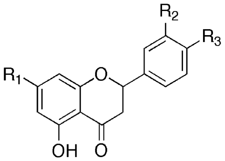
Bergamot has been grown for centuries, albeit in a very limited area of Southern Italy, for the exploitation of its essential oil fraction in perfume industry. Its juice, given its bitter taste, has been considered mainly as an undesired secondary product, and dealt with accordingly, even though folk medicine prescribes a glass a day to maintain a general state of good health. However, recent investigations demonstrated that bergamot, which is presently grown in three different varieties (namely *Castagnaro*, *Fantastico* and *Femminello*), can be regarded as one of the richest in flavonoids content among the *Citrus* juices (*27*, *42*), containing up to 435 ± 16.4, 373 ± 9.5 and 512 ± 12.3 mg/L, for the three varieties, respectively. Their freshly squeezed juices have been analyzed, and they all were found to contain a very wide variety of flavonoid derivatives (Figure 3). The flavonoid fingerprint of bergamot juice present several similarities to those of sour orange or myrtle-leaved orange juices. In fact, naringin **10** (129.8 ± 5.45, 83.5 ± 2.95 and 104.5 ± 4.25 mg/L, for the three varieties, respectively), neohesperidin **11** (89.5 ± 1.85, 80.0 ± 3.1 and 96.4 ± 2.9 mg/L, respectively) and neoeriocitrin **7** (62.6 ± 2.35, 52.2 ± 3.75 and 88.3 ± 4.55 mg/L, respectively) are again among the most abundant of the flavanone *O*-glycosides, even though in this plant the HMG-bearing flavanones, namely melitidin **12** (76.64 ± 11.0, 59.66 ± 6.27 and 58.43 ± 5.75 mg/L, respectively) and brutieridin **13** (147.1 ± 10.3, 157.1 ± 13.8 and 189.0 ± 14.7 mg/L, respectively), are also present in very large amount, with the latter, in particular, being the preeminent component of these juices. Previously, **10**, **11** and **7** were considered to be the most characteristic flavanones in bergamot juice: melitidin **12** and brutieridin **13** have been isolated and fully characterized only recently (*38*).

Table II. Flavanone-*O*-glycosides (7, 9, 13, 16, 17, 18, 21 and 22)



	R_1	R_2	R_3	Structure assignment
5	*O*-Ru[a]	H	*O*-Glu	Naringenin 7-*O*-rutinoside-4´-*O*-glucoside (Narirutin 4´-*O*-glucoside)
6	*O*-Ru[a]	OH	OH	Eriodictyol 7-*O*-rutinoside (Eriocitrin)
7	*O*-Nh[b]	OH	OH	Eriodictyol 7-*O*-neohesperidoside (Neoeriocitrin)
8	*O*-Ru[a]	H	OH	Naringenin 7-*O*-rutinoside (Narirutin)
10	*O*-Nh[b]	H	OH	Naringenin 7-*O*-neohesperidoside (Naringin)
11	*O*-Nh[b]	OH	OMe	Hesperetin 7-*O*-neohesperidoside (Neohesperidin)
12	*O*-Nh(HMG)[c]	H	OH	Naringenin 7-*O*-(6´´-HMG)-neohesperidoside (Melitidin)
13	*O*-Nh(HMG)[c]	OH	OMe	Hesperetin 7-*O*-(6´´-HMG)-neohesperidoside (Brutieridin)

[a] *O*-Rutinose; [b] *O*-Neohesperidose; [c] *O*-(6´´-(3´´´-hydroxy-3´´´-methylglutaryl)) neohesperidose

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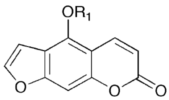
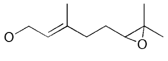
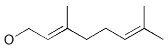
Table III. Furocoumarins (19, 20 and 23)



	R_1	Structure assignment
14		5-(6′,7′-Epoxy)geranyloxypsoralen (Epoxybergamottin)
15	OMe	5-Methoxypsoralen (Bergapten)
23		5-Geranyloxypsoralen (Bergamottin)

Beyond these five flavanones, a striking feature of bergamot juice is the abundance of *C*-glucosyl flavones. Vicenin-2 **2** and lucenin-2 4′-OMe **3**, an apigenin and a diosmetin derivative, respectively, occur in amounts that nearly rival those of the main flavanone components (47.5 ± 1.35 and 25.4 ± 2.0; 44.1 ± 1.0 and 32.7 ± 2.1; 55.2 ± 1.9 and 62.8 ± 3.2 mg/L, for **2** and **3**, in the juices from *Castagnaro*, *Fantastico* and *Femminello* varieties, respectively). On top of those, two other di-*C*-glucosyl derivatives were detected (**1** and **16**), but more interestingly, three mono-*C*-glucosyl flavones are present, namely isovitexin **7**, scoparin **19** and orientin 4′-OMe **20**. In addition, another unique feature of this juice is the presence of *O*- and *C*-glycosyl chrysoeriol derivatives (chrysoeriol 7-*O*-nehosperidoside-4′-*O*-glucoside **17**, chrysoeriol 7-*O*-nehosperidoside **21**, stellarin-2 **16** and scoparin **19**). Lastly, it is worth noticing that also in this case rutinosides are virtually absent, being eriocitrin **6** the sole compound of this type present in bergamot juice. Furocoumarins, in this case, were also found to be present in significant amount: bergapten **15**, 6.7 ± 0.4, 7.6 ± 0.65 and 10.0 ± 1.15 mg/L for the three varieties, but more interestingly bergamottin **23**, found in 27.2 ± 1.55, 23.5 ± 1.05 and 40.2 ± 2.6 mg/L, respectively.

The data here described lend themselves to some deductions. The first immediate observation is that all of the three species under investigation are characterized by the almost exclusive presence of *O*-neohesperidosyl derivatives. This fact has a significant impact on the organoleptic properties of these juices. Flavanone 7-*O*-neohesperidosides, in particular, possess a markedly bitter taste, as opposed to their 7-*O*-rutinoside isomers, which have a neutral taste (*43*).

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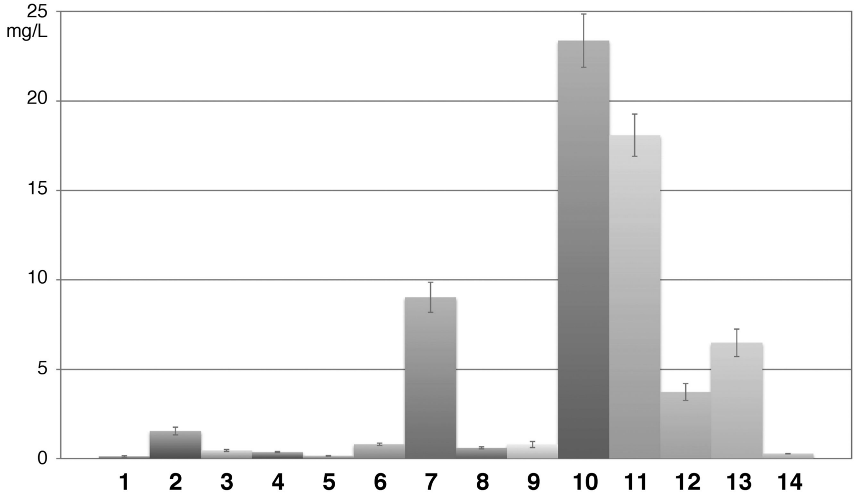

Figure 1. Flavonoids and furocoumarins distribution in Citrus aurantium juice. (Reproduced with permission from reference (36). Copyright 2011 Elsevier.)

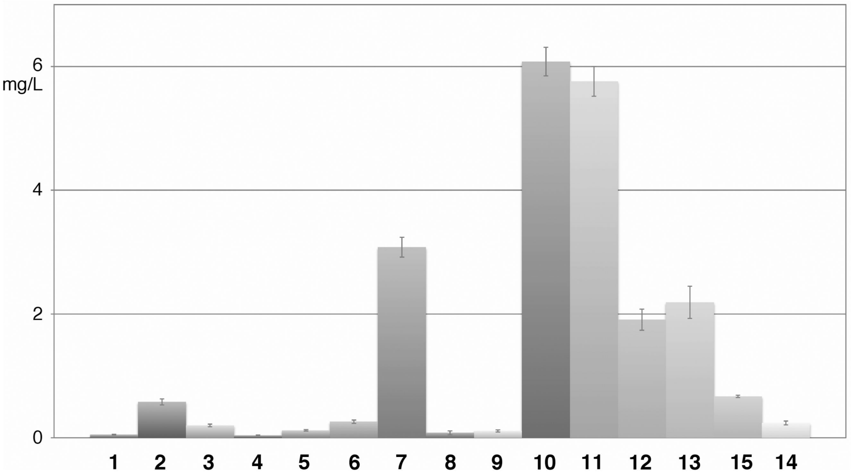

*Figure 2. Flavonoids and furocoumarins distribution in Citrus myrtifolia juice. (With the exception of compounds **1**, **4**, **5**, **12**, reproduced with permission from reference (41). Copyright 2010 ACS.)*

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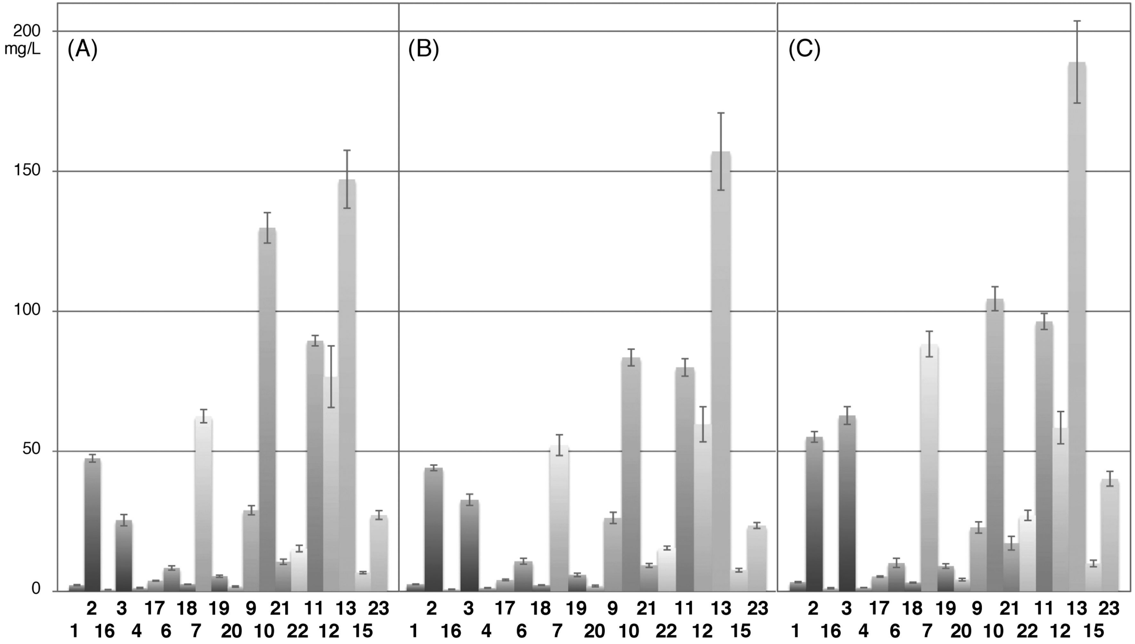

Figure 3. Flavonoids and furocoumarins distribution in Citrus bergamia juices. (A): var. Castagnaro; (B): var. Fantastico; (C): var. Femminello. (With the exception of compounds ***12*** *and* ***13****, reproduced with permission from reference (42). Copyright 2007 ACS.)*

A further observation that can be made is that there is a definite similarity between the flavonoid fingerprints of the three species under investigation. In particular, those for sour orange and myrtle-leaved orange are almost superimposable, the difference in total content notwithstanding. In light of the fact that *C. myrtifolia* is regarded as a mutation of *C. aurantium*, it is noteworthy that the flavonoid fingerprint is retained. Conversely, bergamot, which is, as mentioned, an hybrid of sour orange, along to the components common to the *C. aurantium* group, presents some differences that may be attributed to the contribution of a different genetic parentage. Some groups of compounds appear to deviate from the sour orange-type fingerprint: (i) bergamot contains mono-*C*-glycosides which are totally absent in the juices from the other two species; (ii) the presence of chrysoeriol derivatives (*O*- and *C*-glycosides), which are totally absent in the flavonoid fraction of sour orange and myrtle-leaved orange.

These observations suggest that the flavonoid fingerprint may be regarded by all means as reliable indications of taxonomical relations.

Radical Scavenging Ability

The antioxidant properties of the juices from *C. aurantium*, *C. myrtifolia* and the three varieties of *C. bergamia* were assessed *in vitro* by means of the well-established antioxidant protocol based on 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical quenching (*44*). The assays were carried out directly on crude juice, and the results are reported in Trolox equivalents referred to 1 mL of juice. The results, reported in Table IV, show that these juices as such are powerful antioxidants, with myrtle-leaved orange (which was the one with the lower flavonoid content) slightly less efficient than the others.

To gain a deeper insight into the radical scavenging properties of these complex food matrices, a screening of the antioxidant ability of some selected flavonoids typical of different *Citrus* juices was undertaken, and compared to that of a series of well known commercial and synthetic radical scavengers (*45*, *46*), with the a special attention to the correlation between the glycosylation pattern and the antioxidant activity. The results are depicted in Figure 4.

As shown, rutin **26** (quercetin 3-*O*-rutinoside, a flavonol present in sweet orange), its aglycone, quercetin **25** and ascorbic acid all induce a rapid decrease of DPPH$^{\bullet}$ absorbance, reaching their maximum effect at 25 µM concentration, whereas the flavanone hesperetin **24** (the aglycone of hesperidin **27** and neohesperidin **11**) reaches its highest scavenging activity at 40 µM concentration. Other flavonoids like diosmin **28** (a flavone), naringenin **29** and naringin **10** (flavanones) in these experimental conditions do not show significant radical scavenging activity. Interestingly, hesperidin **27**, a 7-*O*-rutinoside particularly abundant in sweet orange, shows an antioxidant capacity superior to that measured for the isomeric neohesperidin **11**, a 7-*O*-neohesperidoside.

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Table IV. DPPH• Radical Scavenging Ability of the Crude Juices Expressed as μM Trolox Equivalents (TE)

	C. aurantium	*C. myrtifolia*	*C. bergamia var Castagnaro*	*C. bergamia var Fantastico*	*C. bergamia var Femminello*
TE/mL	432.0 ± 5.6	178.0 ± 4.7	396.4 ± 6.1	414.4 ± 3.7	441.2 ± 4.4

Another point worth making regards the comparison of the radical scavenging ability of the tested flavonoids against those determined for commercial and synthetic antioxidants. Ascorbic acid behaves similarly to the most efficient flavonoids (**25** and **26**), whereas widespread synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) fall far behind the efficiency of rutin and quercetin, even though they are still better scavengers than hesperetin and its glycosylated derivatives.

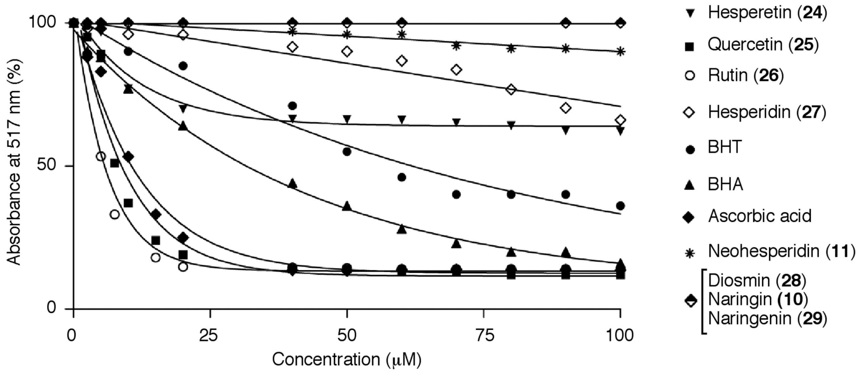

Figure 4. Scavenger activity of tested compounds against DPPH• at 517 nm in the concentration range 0–100 μM. (Reproduced with permission from references (45) and (46). Copyright 2009 Springer.)

These results, along with data from literature sources (*47*), provide useful information to discuss the structure-activity relations in the flavonoids found in the three species here investigated. The flavonoid fractions of sour orange, myrtle-leaved orange and bergamot juices are exclusively composed of flavanones and flavones. The derivatives responsible for most of the antioxidant activity of the crude juices (along to other classes of compounds, such as ascorbic acid, of course (*48*)) are most probably the flavonoids possessing a cathecolic B-ring (*49*), such as neoeriocitrin (**7**), eriocitrin (**6**), lucenin-2 (**1**, which also possesses a wider conjugated system owing to the double bond in the 2,3 position of the C-ring), given that the presence of a cathecol moiety confers greater stability to the aroxyl radicals (*47*, *50*) formed upon H• abstraction by DPPH• radicals (*51*). The rest of the flavonoids pool presumably provides a relatively smaller contribution to the total antioxidant capacity. In fact, even though they still possess significant radical scavenging ability, they all present structural features that slightly decrease their reactivity toward DPPH• radicals, examples being the substitution at the 4′-position on a cathecolic B-ring (as in **3**, **11**, **13**, **20** and **22**), which destabilizes the aroxyl radicals (*50*), or the glycosylation on the 7-OH position on a flavanone skeleton (as in most of the *O*-glycosyl flavonoids found), which shows a suppressive influence on the antioxidant activity (*45*).

Antiproliferative Activity and Signal Cascade Activation

Citrus juices, as mentioned above, are a rich source of health-promoting compounds, with a broad spectrum of biological activities (including anti-carcinogenic and anti-proliferative action), along with the capability of preventing some types of carcinogenesis. These actions likely depend on the antioxidant properties of these compounds, which in turn are a consequence of the nature of the aglycone and on the type and position of the glycosyl substituents.

The influence of these structural features was revealed investigating the antioxidant and antiproliferative properties of three correlated flavonoids, such as hesperetin **24**, hesperidin **27** and neohesperidin **11**, which differ only for the presence/absence and the type of the disaccharide unit (i.e. no sugar, a rutinose or a neohesperidose, respectively). These compounds were tested towards hepatoma cell lines (Hep G2) in incubation experiments for 48 h (*46*). Flow cytometric analysis (Figure 5) showed that the glycosylated derivatives were able to inhibit Hep G2 cell growth up to 68.4 % (in the case of **11**) and 64.6 % (in the case of **27**), with a significant increase (up to 1.40 and 1.33-fold, respectively) with respect to their aglycone hesperetin **24**, which nonetheless showed a good antiproliferative effect (48%).

The inset in Figure 5 shows the percentage of cells alive or dead in synchronized adherent Hep G2 cells after 48 hours of treatment with the three flavonoids, added at a concentration of 100 μM. In the presence of neohesperidin **11** and hesperetin **24**, 37 and 28% of total cell population was dead, whereas the differences between hesperidin **27** and control are negligible.

These experiments demonstrate the antiproliferative activity of these molecules, but do not provide information on their cytotoxic effects. To this end, fluorescent microscopy, carried out with acridine orange as a staining agent, allowed us to analyze the cytotoxicity and the morphological features resulting from neohesperidin **11**, hesperidin **27** and hesperetin **24** treatment of Hep G2 cells. In the case of the aglycone **24**, a significant amount of cells with membrane babblings, condensed chromatin bodies and DNA fragmentation can be easily detected, whereas very few or no apoptotic cells were found in the presence of the glycosylated forms **11** and **27** (Figure 6).

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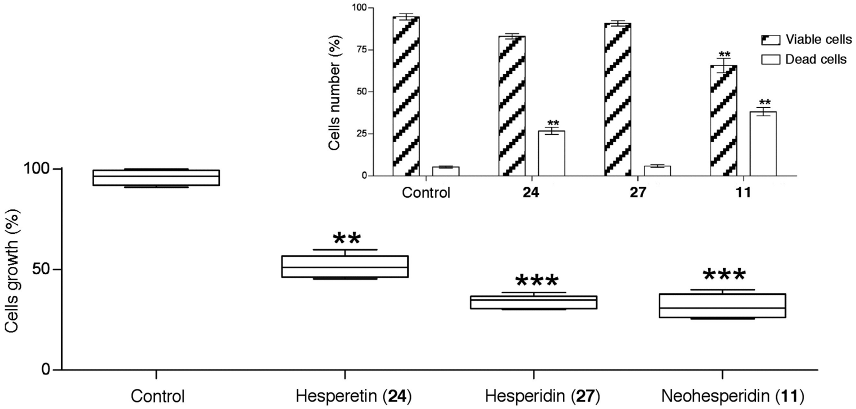

*Figure 5. Antiproliferative effects of flavonoids **11**, **24** and **27** (100 µM) against Hep G2 cell line. Inset: cytofluorimetric determination of viable and dead cells. (Reproduced with permission from reference (46). Copyright 2009 Springer.)*

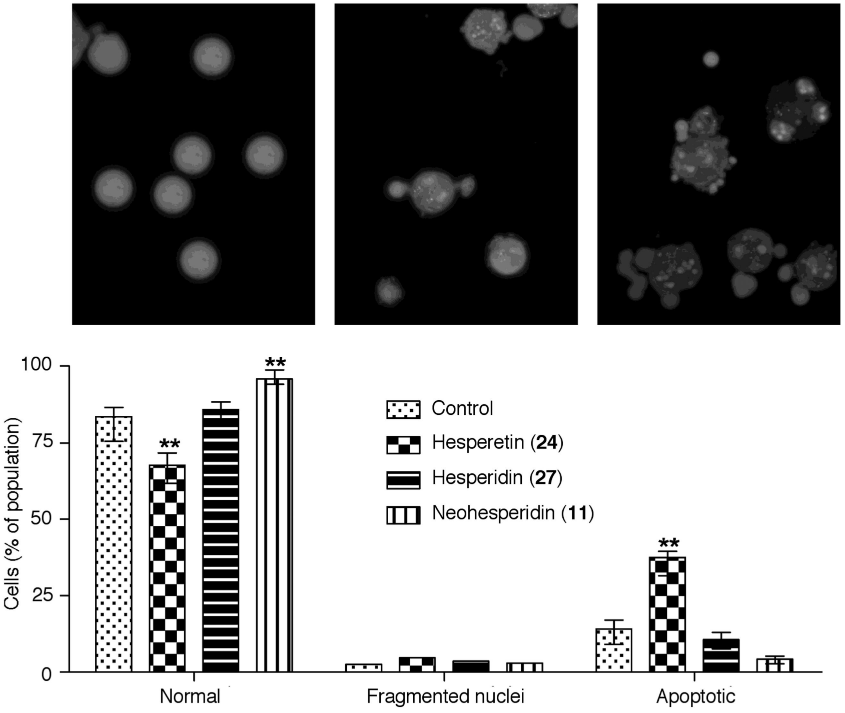
100
75
50
25
0
Cells (% of population)
**
**
**
Control
Hesperetin (24)
Hesperidin (27)
Neohesperidin (11)
Normal
Fragmented nuclei
Apoptotic

*Figure 6. Bottom: morphological analysis by fluorescence microscopy of Hep G2 cells cultured in presence or absence of 100 µM flavonoids **11**, **24** and **27** (48 h). Top: morphological features of cells analyzed. (Reproduced with permission from reference (46). Copyright 2009 Springer.)*

In addition, experiments on the dependence of apoptotic pathway activation due to caspase-3 were carried out. Apoptosis is a type of cell death regulated by a number of genes that promote apoptosis or cell survival, and it is activated in response to external or internal noxious stimuli. Flavonoids, besides acting as antioxidant species, exert a modulatory action on cell signaling pathways, altering the phosphorylation state of the target molecules and modulating gene expression. Again, glycosylation of the flavonoid was found to markedly influence the response of the compounds tested. Inspection of the cultured cell samples showed that hesperetin **24** activated caspase-3, whereas no significant variation was detected in the presence of the *O*-rutinosyl or *O*-neohesperidosyl derivatives (Figure 7).

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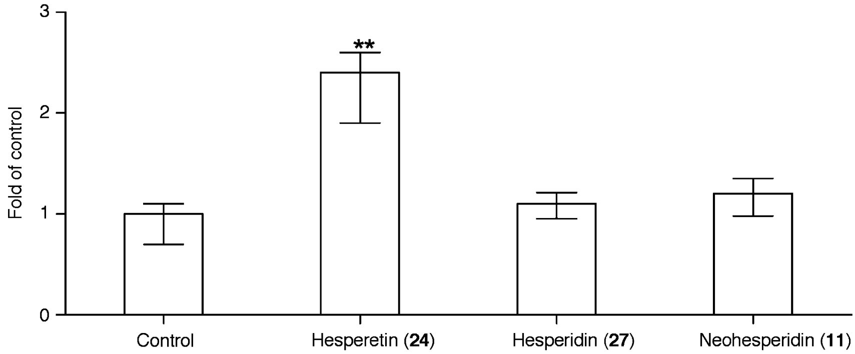

*Figure 7. Caspase-3 activity in Hep G2 cells cultured for 48 h in presence of 100 μM flavonoids **11**, **24** and **27**. (Reproduced with permission from reference (46). Copyright 2009 Springer.)*

The flavonoids under investigation do not merely induce morphological changes and alteration of the cell signal pathways, but also markedly influence cell cycle, an effect that may account for the anticarcinogenic and antiproliferative effects of flavonoids. Mitogenic signals induce cells to proceed through the series of regulated steps of the cell cycle. Synthesis of DNA (S phase) and separation of two daughter cells (M phase) are the main step of cell cycle progression. The period elapsing between the S and M phases(the G2 phase) is important, since cells may fix errors occurred during DNA duplication. In contrast, the G1 phase is the period that separates M and S phases during which cells prepare for DNA duplication. The effects of the three flavonoids on Hep G2 cell cycle progression are shown in Figure 8. Our results indicated that, in synchronized Hep G2, the cell population is almost entirely in the G0/G1 phase. After 48 hours from the treatment, the inspection of the samples indicated that about 87, 90, and 94 % of cells were in G0/G1 phase in the presence of 100 μM neohesperidin **11**, hesperidin **27** and hesperetin **24**, respectively. The G2/M phase cells are comprised between 0.7 and 3%, whereas those in S phase between 5 and 12%. It should be added that

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a modification of the polyamine biosynthesis could be an effect that contributes to the G0/G1 block induced by flavonoids. The different action of the tested flavonoids might be also related to their influence on the polyamines internal pool (data not shown (*46*)). These endogenous polycations (spermidine, spermine and putrescine) have a fundamental role in cellular proliferation and differentiation, and their level is highly regulated by the enzymes involved in their synthesis and catabolism (*52*).

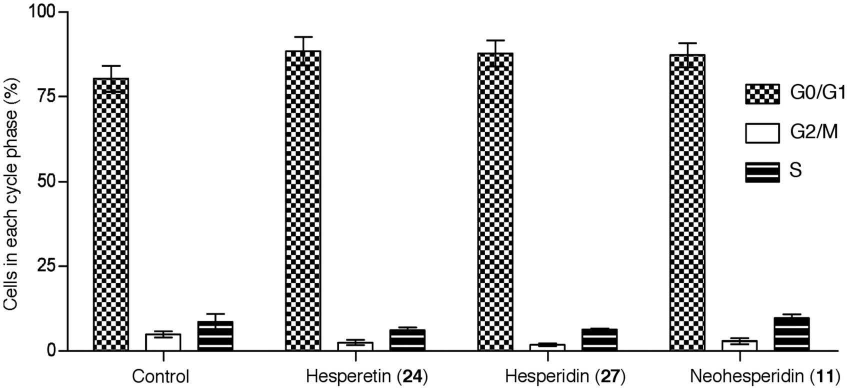

Figure 8. Cell cycle distribution of flavonoids-treated (100 μM, 48 h) Hep G2 cells determined by flow cytometric analysis. The percentage of cells in G0/G1, S and G2/M phases are represented as percent of total population. (Reproduced with permission from reference (46). Copyright 2009 Springer.)

Conclusions

The data here discussed lead to few different conclusions. Firstly, it has been shown how the methodology we developed, which is based on HPLC-DAD-ESI-MS-MS analysis, allows for the direct identification and quantification of an unprecedented number of flavonoid and furocoumarin components, by means of a single chromatographic course, injecting the crude juice without any preliminary extraction.

The second key point regards the use of the flavonoid fingerprint to reveal relationships between different taxons, as it has been demonstrated in the present study on three species belonging to the *Citrus* genus. To this end, data on sour orange, myrtle-leaved orange and bergamot, which are similar in their flavanone/flavone profiles, were aggregated and presented together. Future extension of these studies may provide the means to elucidate the taxonomical origin of many disputed species, as well as providing a powerful tool to detect frauds and adulterations of *Citrus*-based food products.

Finally, the results on the antioxidant capacity tests, carried out both on juices as such and on selected flavonoids, and the studies of Hep G2 cells clearly demonstrate the potential of these naturally occurring compounds to inhibit hepatoma cells proliferation blocking cell cycle progression. This behavior, along with the very low cytotoxicity displayed by the *O*-glycosylated flavonoids investigated, open up the way for future research aimed at the use of these remarkable compounds in therapeutic treatments.

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Chapter 3

Cancer Chemopreventive Properties of Citrus Limonoids

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Limonoids constitute one of the major phytochemicals along with flavonoids, coumarins, and carotenoids in citrus. Limonoids were shown to possess cancer preventive properties. In the past quarter century, a number of studies have accumulated considerable evidence, which advanced our understanding about the disease prevention mechanism of limonoids. It is speculated that limonoids suppress cell proliferation and induce apoptosis by inhibiting enzyme activities and signal transduction pathways. In addition, limonoids were also shown to inhibit cell metastasis in cell culture and animal studies. However, further research is required to identify the cellular targets of active limonoids. Identification of the target mechanism at cellular level may result in the development of limonoids as anticancer agents. In the present chapter, the diverse role and the biological activities of limonoids relating to their potential in cancer prevention are discussed.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Introduction

Citrus limonoids are a class of secondary metabolites known as triterpenoids, and act as a defense agents against insects. Until now, 62 limonoids, including 44 aglycones and 18 glucosides, have been identified from citrus fruits, and still the number is increasing (Table 1) (*1*). The early research focused on the identification of limonoids because of the delayed bitterness caused by certain limonoids such as limonin and nomilin. Limonin and other bitter limonoids were considered as a undesirable component to maintain the acceptable-quality level for the fresh or processed juice industry. However, recent research has discovered the various health benefits and pharmacological uses of limonoids such as antibacterial, antifungal, antiviral, antioxidant, and anticancer (*2–4*).

Biosynthesis of Citrus Limonoids

Triterpenoids are the largest group of plant secondary metabolites, and over 4000 triterpenoids have been identified in the plant kingdom (*6*). The limonoids are biosynthesized in the cytosol and distributed in different parts of plant such as seed, stem bark, leaf, and fruit (*7*). The biosynthesis occurs based on the “biogenetic isoprene rule” (*8*). Isopentenyl diphosphate (IPP) and its allylic isomer dimethyl allyl diphosphate (DMAPP) are common precursors of isoprene biosynthesis. The limonoids are synthesized *via* a mevalonate pathway to produce triterpenoids (*9*). The triterpenoids consist of 30 carbons and are derived from squalene (*10*). Limonoids are degraded triterpenoids, also called tetranotriterpenoids, consist of a furan ring attached at C-17 and contain a functional group at C-3, C-4, C-7, C-16, C-17, and C-19 (*2*, *10*). The limonoids are synthesized by squalene through oxidation and the rearrangement process and the basic skeleton come from euphol or tirucallol, which are considered precursors of limonoids (*10*) (Figure 1).

By using radioactive tracer techniques, citrus metabolites were shown to possess two distinct precursors during limonoid biosynthesis. In principle, deacetylnomilinic acid is a precursor of limonoids in *Citrus* which gets converted to nomilin in the phloem region of the stem (*11*). In addition, stem tissue synthesizes nomilin directly from acetate (*7*). The synthesized nomilin is translocated from the stem to other parts of the plant, such as leaves, fruit, seed, and root tissues. Other limonoids are biosynthesized from nomilin in all the region of the plant except the cortex and inner core (*7*).

Based on structural diversity and biosynthetic origin, limonoids are divided into 4 different groups—limonin, calamin, ichangensin, and 7α-acetate limonoids (*12*) (Figure 2).

Citrus limonoids are present in the glucoside or aglycone. The aglycones converted to glucosides by the UDP-D-glucoside: limonoid glucosyltransferase enzyme during the fruit maturation process (*13*). Higher concentration of total limonoids were found in the seeds as compared to fruits, suggesting that seeds act as sink for these compounds (*14*). However, the glucosides concentration are less than half of the aglycones in the seed (*14*).

Table 1. Identified limonoid aglycones and glucosides from different species and its closely related genera*

	Aglycones	Glucosides
Limonin Group	17-dehydroxylimonoic acid A-ring lactone	Deacetylnomilin glucoside
	19-Hydroxydeacetylnomilinic acid	Deacetylnomilinic acid glucoside
	7α-Obacunnol	Deoxylimonic acid glucoside
	Deacetylnomilin	Epiisoobacunoic acid glucoside
	Deacetylnomilinic acid	Ichangin glucoside
	Deoxylimonic acid	Isolimonic acid glucoside
	Deoxylimonin	Isoobacunoic acid glucoside
	Deoxylimonol	Limonin glucoside
	Epiisoobacunoic acid	Nomilin glucoside
	Ichanexic acid	Nomilinic acid glucoside
	Ichangin	Obacunoic acid glucoside
	Isolimonexic acid	Obacunone glucoside
	Isolimonic acid	*trans*-Obacunoic acid glucoside
	Isoobacunoic acid	
	Isoobacunoic acid diosphenol	
	Limonexic acid	
	Limonin	
	Limonol	
	Nomilin	
	Nomilinic acid	
	Obacunoic acid	
	Obacunone	
Calamin Group	6-Keto-7β-deacetylnomilol	19-Hydroxydeacetylnomilinic acid glucoside
	6-Keto-7β-nomilol	6-Keto-7β-deacetylnomilol glucoside
	Calamin	Calamin glucoside
	Calaminic acid	Methyl deacetylnomilinate glucosdie
	Cyclocalamin	
	Cyclocalaminic acid	
	Isocyclocalamin	
	Methyl deacetylnomilinate	
	Methyl epiisoobacunoate	
	Methyl isolimonate	
	Methyl isoobacunoate	
	Methyl isoobacunoate diosphenol	
	Methyl nomilinate	
	Methyl obacunoate	
	Methyl-*trans*-obacunoate	
	Retrocalamin	
	Retrocalaminic acid	
Ichangensin Group	Ichangensin	Ichangensin glucoside
7α-Acetate Limonoid Group	1-(10-19)abeo-7α-acetoxy-10β-hydroxyisoobacunoic acid 3,10-lactone	
	1-(10-19)abeo-obacun-9(11)-en-7α-yl acetate	
	7α-Obacunyl acetate	
	Limonyl acetate	

* Referred from (*1, 2, 4, 5*)

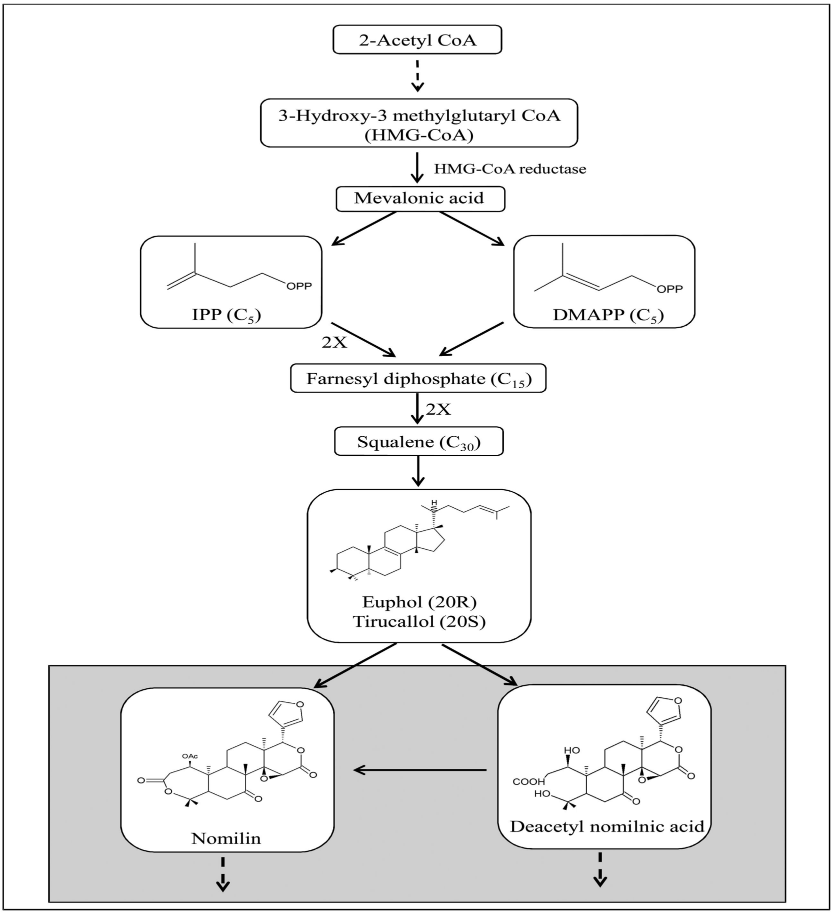
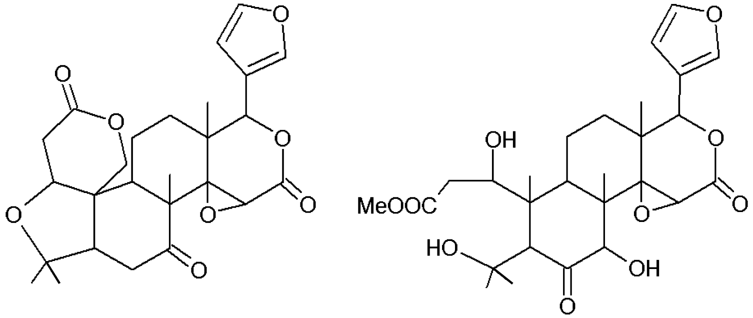

2-Acetyl CoA
3-Hydroxy-3 methylglutaryl CoA (HMG-CoA)
HMG-CoA reductase
Mevalonic acid
OPP
IPP (C_5)
OPP
DMAPP (C_5)
2X
Farnesyl diphosphate (C_{15})
2X
Squalene (C_{30})
H
Euphol (20R)
Tirucallol (20S)
OAc
O
O
O
O
O
O
O
Nomilin
HO
COOH
HO
O
O
O
O
O
Deacetyl nomilnic acid

Figure 1. The biosynthesis of citrus limonoids occurring in the cytosol via mevalonate pathway. IPP, Isopentenyl diphosphate; DMAPP, dimethyl allyl diphosphate.

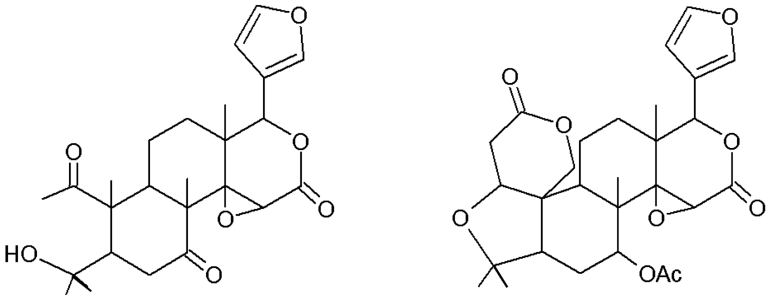
Stability and Bioavailability of Citrus Limonoids

Citrus fruits are rich in health-promoting constituents. In addition to ascorbic acid, citrus contains flavonoids, carotenoids, coumarins, volatile oil, sitosterols, pectin, and limonoids (*3*, *15*). Limonoids and flavonoids are present as glucosides as well as aglycone form. The solubility and bioavailability are often considered an important factor to improve the health beneficial effects of bioactive compounds. Accordingly, the identification of digestive metabolites, intracellular metabolism, plasma transport, and toxicity have been intensively studied for understanding the bioavailability properties (*2*, *16*, *17*). The limonin and limonin glucoside are the predominant limonoids in orange juice, and the limonoid glucosides concentration ranges between 350 and 400 ppm (*18*). Due to the bitterness of limonin, the amount of limonin is considered an important quality attribute of the citrus processing industries. The bitterness threshold of limonin in orange juice is 6.5 ppm, and the threshold is maximized at pH 3.8 because the optimum pH suppresses the limonin bitterness (*19*). The stability and degradation of limonin during juice processing need to investigated. The thermal properties of limonin is very stable and the melting point is 298°C (*20*). The stability and decomposition rate of compounds could be affected by several factors such as humidity, temperature, pH, and oxygen level. Limonin is stable over a wide range of pH value (pH 2-9) and most stable at pH 5 at 45 ºC (*18*). However, the limonin is completely degraded at pH 10-12. On the other hand, the limonin produces isomer named limonexic acid due to attack on the furan ring with strong acids (*20*). Moreover, the limonin stability was affected by temperature in acidic and basic conditions. The limonin exhibited maximum stability at 45ºC compare to 70-80 ºC (*20*).



Limonin
(Limonin Group)

Calamin
(Calamin Group)



Ichangensin
(Ichangensin Group)

7α-Limonyl acetate
(7α-Acetate Limonoid Group)

Figure 2. Classification of limonoids of citrus and its related genera.

Several studies have been focused on understanding the bioavailability and toxicity of natural products due to the chemopreventive efficacy depending on its absorption, metabolism, and safety (*21*, *22*). However, only one study was conducted on the bioavailability of limonoids till date. The limonin glucoside, upto 2 g/per day, was fed to the subjects and metabolites were measured in the plasma using LC-MS technique (*4*). The results of the study suggested that the limonin glucoside was converted into limonin as final metabolite with maximum plasma concentration at 6 h (*4*). During the experiment, an unidentified peak was also detected indicating presence of an additional metabolite. The unknown peak was later identified as epilimonin, an isomer of limonin (*23*). Although there are limited studies on absorption of citrus limonoids in the human body, investigation using soy phytochemicals suggest that aglycones show much faster and higher absorption rates than glucosides in humans (*24*).

In addition, several toxicity studies were reported using animal models. Miller *et al.* (*16*) investigated the long term feeding of limonoids mixture on weight gain in pregnant rats. These results demonstrated that diet supplement with 0.25% of limonoids caused a significant reduction in weight gain in female progeny (*16*). Furthermore, male progeny was affected by 0.15% of mixture of limonoid aglycones and 0.25% mixed limonoid glucosides in the feed (*16*). Even though the results suggest the exposure of high concentration of limonoids causes problem with weigh gain, such a correlation needs to determined in humans. For example, daily consumption 130 glasses of orange juice for one week is required to reach a similar level for a 60 kg adult (*16*).

Cancer Chemoprevention by Citrus Limonoids

A significant effort has been spent in the last century for studying the health beneficial properties of citrus bioactive compounds (*1*, *25–31*). More than 290 bioactive compounds have been reported so far in citrus species, including 115 carotenoids (*32*), 60 flavonoids (*32*, *33*), 57 limonoids (*31*), 31 volatile oils (*34*), 17 coumarins (*35*), and 6 amines (*36*). Due to the presence of these compounds, citrus is considered one of the promising healthy foods. For instance, limonoids have shown strong activity to suppress tumor formation in a various cancers, reduce risks of heart disease, neurodegenerative disease, and autoimmune disease (*3*, *17*, *37*, *38*). Among the chronic diseases, cancer is the most common malignant, and more than 13% of human deaths (7.6 million) are associated with cancer in the world. In USA, approximately 560,000 Americans, more than a quarter-percent of the USA population die from cancer (Table 2) (*39–41*). However, the complexity of the disease, mortality, and morbidity of cancer, the mechanism of tumor formation and resistance to therapy is poorly understood. Interest in preventive therapies using a nutritional and physical approach is now increasing as these alternative approaches may help in delaying the progression of carcinogenesis.

Table 2. Estimated Mortality due to Chronic Diseases in the United States

Cause of Death	*1986*			*2007*			*Ref.*
	Rank	*Deaths*	*(%)*	*Rank*	*Deaths*	*(%)*	
Heart Diseases	1	765,490	36.4	1	616,067	25.4	(*41*, *42*)
Cancer	2	469,376	22.3	2	562,875	23.2	
Cerebrovascular diseases	3	149,643	7.1	3	135,952	5.6	
Chronic lower respiratory diseases	5	76,559	3.6	4	127,924	5.3	
Accidents	4	95,277	4.5	5	123,706	5.1	

Limonoids have been identified for their anti-carcinogenic activity in cell cultures and animal model systems (Table 3).

The possibility of an anti-carcinogenic property of limonoids from citrus species is being actively investigated in the colon cancer. Glutathione S-transferases (GST) are an important catalytic and binding protein family due to its ability to detoxify carcinogens (*55*). In 1989, Lam and coworkers reported the induction of GST enzyme in small intestinal mucosa by nomilin and limonin (*56*). In another study, while limonin showed minimal effect, nomilin increased the GST activity three times compared to the control group. Furthermore, recent study from our lab demonstrated that a limonin analogue, limonin-7-methoxime was a potent inducer of GST in mice (*44*). Further, in 1994, it is demonstrated that limonin and nomilin suppress chemically induced tumor size in an animal model (*57*). Additionally, obacunone and limonin have shown the ability to inhibit formation of aberrant crypt foci (ACF) in azoxymethane (AOM) induced colon cancer model in rats (*47*). After 4 years, in 2005, limonin demonstrated the suppression of chemically induced ACF formation in similarly designed animal experiment (*28*). Based on the results, the authors speculated that the possible mechanism may be apoptosis mediated by down-regulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are involved in the inflammatory process (*28*). Furthermore, limonoid aglycones such as limonin, nomilin, obacunone, and deacetyl nomilin showed antiproliferative activity through induction of caspase 3/7 in colon carcinoma Caco-2 cells (*27*). In addition, ichanexic acid and isolimonexic acid inhibited the G2/M phase, inducing cell cycle arrest in human colon adenocarcinoma HT-29 cells (*1*). More recently, limonexic acid was shown to induce cell cycle arrest in human colon adenocarcinoma HT-29 cells (*26*). Taken together, these results indicated that cell cycle arrest and apoptosis through inhibition of iNOS/COX-2 expression and induction of caspase 3/7 might be considered a mechanism by which limonoids may exhibit chemopreventive property in colon cancer.

Table 3. Mechanisms of Chemopreventive Properties of Citrus Limonoids in Different Type of Cancer

Target Organs	**Limonoids**	**Possible Mechamisms and Functions**	**Ref.**
Colon	Limonin	Inhibition of P-glycoprotein	(*42*)
		Suppression of iNOS and COX-2	(*28*)
		Induction of detoxifying enzyme	(*43-46*)
		Inhibition of AOM-induced ACF	(*28, 47*
		Apoptosis	(*48*)
	Limonin glucoside	Apoptosis	(*48*)
	Limonexic acid	Anti-proliferation	(*26*)
		Cell cycle arrest	(*26*)
	Nomilin	Induction of GST anad QR	(*46*)
	Methyl nomilinate	Cell cycle arrest	(*49*)
	Obacunone	Inhibition of AOM-induced ACF	(*50*)
		Induction of GST anad QR	(*46*)
Breast	Deacetyl nomilin Limonin Limonin methoxime Methyl nomilinate Nomilin Nomilinic acid glucoside Obacunone Obacunone glucoside	Anti-proliferation	(*29, 51*)
Liver	Limonin	Reduction of ApoB production	(*52*)
		Induction of GST	(*50*)
		Inhibition of BP-treated tumor size	(*50*)
	Nomilin	Induction of GST	(*50*)
Brain	Deacetyl nomilin Limonin Nomilin Obacunone	Anti-proliferation	(*27*)
	Limonin 17-β-D-glucopyranoside Obacunone glucoside	Apoptosis	(*53*)
Pancreas	Isolimonexic acid	Apoptosis	(*1*)
	Limonin		(*31*)
	Limonin 17-β-D-glucopyranoside		(*31*)
	Limonexic acid		(*31*)
	Obacunone		(*30*)
Mouse	Deoxylimonin Limonin 17-β-D-glucopyranoside Limonin carboxymethoxime Nomilin Obacunone	Reduction of tumor burden	(*54*)
Blood	Limonin	Inhibition of P-glycoprotein	(*42*)
Lung	Limonin	Inhibition of BP-treated tumor size	(*50*)
	Nomilin	Inhibition of DNA methylation	
Stomach	Limonin Nomilin	Inhibition of BP-treated tumor size	(*50*)

Limonoids were reported to inhibit the cell proliferation in MDA-MB-435 estrogen receptor-negative (ER-) and MCF-7 estrogen receptor-positive (ER+) breast cancer cell lines (*29*, *51*). However, the anti-proliferative activity by limonoids in breast cancer cells and the mechanism of cell growth inhibition by limonoids remains to be elucidated. Our recent and other studies reported that limonin and nomilin could inhibit cancer development in the neuroblastoma (*27*, *53*), pancreatic *in vitro* (*31*), stomach, lung, and skin *in vitro* and *in vivo* (*37*). Recently, our study also showed that obacunone induces apoptosis and inhibits inflammatory markers in pancreatic cancer Panc-28 cells (*30*).

Human cytochrome P450 oxidoreductase enzymes (CYPs) known to involve in drug metabolism, degradation of xenobiotics, and detoxification processes (*58*). Several studies have demonstrated that the overexpression of the CYPs has strongly associated with cancer susceptibility (*58*). The limonoids were evaluated as potent inhibitor of CYPs (*59*, *60*). Fukuda *et al.* (*59*) found that the limonin and obacunone were potent inhibitor of CYP3A4 in rat and human liver mircosom. Furthermore, our recent study also indicated that nomilin, NAG, and LG is a potent inhibitor of CYP3A4 activity (*60*). However, the specific interaction of limonoids and CYPs is remained to be elucidated.

The structure-activity relationship of bioactive compounds is an important parameter for understanding the biological activity. Although the structure-function relationship of limonoids for cancer inhibitory activity has not been studied, our results suggest that the presence of furan and an intact A ring structure are responsible for chemopreventive activity in cancer cells. For example, furan ring seems to play an important role in anti-proliferative activity of Panc-28 cells (*31*). In particular, the configuration of furan ring is critical for action. This is exemplified by the difference in activities of isolimonexic acid and limonexic acid, which differ in the configuration of furan ring. Other critical factors seems to be A-ring and glucose moiety. A-ring also seems to play important role in anti-proliferative activity of limonoids against MCF-7 cells (*29*). Specifically, presence of 7-membered A-ring (obacunone and nomilin) imparts higher activity compared to presence of A and A′ ring present in limonin.

Other Biological Activities of Citrus Limonoids

Limonoids exhibited anti-HIV, moderate radical scavenging, and anti-oxidant activity (*53*, *61*). Recent studies have demonstrated the anti-microbial, antibacterial, and antifungal activity of limonoids in the khaya species, as well as in several other plants found in the *Meliaceae* and *Rutaceae* family (*2*). For the first time, our study reported that certain citrus limonoids inhibit bacterial cell-cell signaling and biofilm formation (*62*, *63*). Specially, obacunone was the most potent inhibitor of *Escherichia coli* O157:H7 biofilm formation (*63*). Michael *et al.* reported the structure-activity relationships of limonoids on antifeedant activity (*64*). The results demonstrated that the furan ring and the epoxide group have the highest activity against insects (*64*). Furthermore, studies have shown that the A ring of the limonoid nucleus may be a key regulator for antineoplastic activity and cancer chemopreventive activity (*43*, *45*) whereas, the D ring may not be associated with biological activity (*17*, *65*). Therefore, the structure-function activity of limonoids plays a vital role in understanding their biological action. More extensive research is required to understand the mechanism of proliferation inhibition by structurally related limonoids.

Conclusions

The research on the diverse role of limonoids on human cancer prevention is still ongoing. Citrus is a unique source for limonoids which may potentially reduce risk from certain types of cancers. However, future research is needed to understand the bioavailability and metabolism of the agents. In addition, incorporation of epidemiological study based on solid experimental results will contribute significantly on the role of limonoids as chemopreventive agents.

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Chapter 4

Isolation and Chemical Characterization of Components with Biological Activity Extracted from *Azadirachta indica* and *Melia azedarach*

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In this chapter we review the biological activity of *Azadirachta indica* A. Juss (neem) (AI) and *Melia azedarach* L. (chinaberry) (MA), as well as the principal components this activity has been attributed to. We develop analytical methods to elucidate the structure and to quantify the most abundant limonoids contained in seeds of AI and MA and report on their biological properties. The limonoids obtained from AI were azadirachtin A and B, nimbin, salannin and their acetyl derivatives; while from MA we obtained a new triterpenoid, named 3-α-tigloylmelianone, as well as three known limonoids named methyl kulonate, 21-β-acetoxymelianone and melianone. The elucidation of the limonoids' structures was performed by means of 1D and 2D-NMR and the accuracy of theoretical exact mass values, was deduced by HPLC-Q-TOF analysis. According to our results 21-β-acetoxymelianone and 3-α-tigloylmelianone show both antiproliferative (IC_{50} = 100 and 91.8 µg/mL) and cytotoxic (CC_{50} = 90.6 and 64.7 µg/mL) activity on the tumorigenic cell line A549, while melianone exhibited the highest cytotoxic activity with a CC_{50} calculated at 3.6 µg/mL. Methyl kulonate as well as limonoids isolated from AI were

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

found neither cytotoxic nor antiproliferative. Methanol extract from MA fruits was analysed by GC/MS and found to contain hexadecanoic, acetic, and hexanoic acids as well as furfural and 5-hydroxymethylfurfural. The extract was found nematicidal on *Meloidogyne incognita*, both in terms of juveniles paralysis ($EC_{50/96h}$ = 0.04 %) and biological cycle arrest (EC_{50} = 0.91 %). This activity, in the concentration range of 31.2-500 µg/mL, has been attributed to the organic acids and aldehydes present and not to the limonoids. Furfural exhibited the highest nematicidal activity for both immersion in test solutions and exposure to vapours ($EC_{50/1\ day}$ = 8.5 and 24.1 µg/mL, respectively).

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Introduction

Plant allelochemicals are products of species coevolution with many environmental advantages that have incorporated them into crop protection and pharmacy (*1*). The role of these compounds in the plant organism is of ecological nature since they do not contribute to the basic cell metabolism and are not essential to the growth, development and reproduction of the plant. Plant allelochemicals are the defence mechanisms against predators and they comprise various substances such as limonoids, quassinoids, saponins, terpenes, alkaloids, organic acids and phenols. Limonoids represent a large group of plant allelochemicals, they are found most abundantly in members of the plant families Meliaceae and Rutaceae of the order Rutales (Sapindales). The term limonoid is derived from limonin, the first tetranortriterpenoid obtained from citrus fruit by Highby in 1938. They are highly oxygenated, modified terpenoids, with a prototypical structure either containing or derived from precursor with a 4,4,8-trimethyl-17-furanylsteroid skeleton. Citrus limonoids contain a furan ring attached to the D-ring, at C-17, as well as oxygen containing functional groups at C-3, C-4, C-7, C-16 and C-17. Most of the biogenetic proposals are tentative as they are not supported by valid biosynthetic studies.

The structural variations of the limonoids found in Meliaceae are more than those found in Rutaceae with a high degree of oxidation and rearrangement exhibited in the parent limonoid structure. Over 300 limonoids are currently known, and about a third of them are found in the species AI and MA. Azadirachtin A [1] is thought to be the most useful and fascinating by-product of AI (*2*). Azadirachtin based products exhibit good efficacy on more than 400 insect species (*3*, *4*). Significant activity has also been exhibited by limonoids from MA. MA is a large evergreen tree native to India, but currently growing in a number of continents including Europe. It is very similar to AI and it has outstanding phytochemical and pharmacological uses (*5*). Azadirachtin content in MA suffers from strong geographical dependence (*6*). According to recent studies when AI is grafted on MA rootstock it produces azadirachtin as well as fruits free from meliatoxins; this forms an excellent basis for breeding vigorous AI trees which can grow in cooler regions (*7*). Limonoids isolated from various

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Meliaceae species exhibit a range of pharmacological and biological activities like antioxidant (*8*), cytotoxic (*9–14*), anti-inflammatory (*15*), antimalarial (*16*), anthelmintic (*17*, *18*), antibacterial (*19*), antifungal (*20–22*), antiviral (*23*), nematicidal (*24*, *25*) and insecticidal, on disease vectors and domestic insects (*26*, *27*) as well as other insect pests and especially *Spodoptera* sp. (*4*, *28–36*). From an ecological standpoint these products do not disturb ecosystems because they are not toxic to natural enemies of pests (parasitoids and predators) (*37*) and they have a relatively short residual life (*38–40*). Nevertheless, at high concentrations they can be poisonous to humans (*41*). Their insecticidal properties are based on repellence, anti-feeding action due to destruction of the function of chemoreceptors, contact or stomach poisoning and growth inhibition, as well as larval stage prolongation due to reduced food consumption and weight gain (*42*). Studies of the relationship between structure and activity have shown that limonoids with an intact apoeuphol skeleton, a 14, 15 β epoxide, and a reactive site such as either a 19—28 lactol bridge or a cyclohexanone 'A' ring are biologically very active. C-seco limonoids with an enone system in ring 'A' are potent cytotoxic and anti-malarial agents; they are two to three times more active than other limonoids and highly active against herbivorous insects. The 'A' ring in the limonoid nucleus may be critical to the antineoplastic activity. The hydroxyl group of azadirachtin and derivatives is correlated to the insect feeding inhibitory activity; the free hydroxyls of azadirachtin contribute to the insect growth-inhibitory effects; the hydroxydihydrofuran portion of the azadirachtin molecule is responsible for up to 50-60% of the feeding deterrent properties. The furan ring and epoxyde groups of limonin are important for the antifeedant properties (*2*). Some of the limonoids isolated from MA are presented in Table 1. Limonoids obtained from AI have not been included herein since various relevant literature reviews are currently available (*43–46*). The activity of AI and MA has not been always attributed to their limonoids' content. The aim of this study was to review the international bibliography reporting on biological activity of extracts or purified compounds obtained from the two species; and to present the chronology of limonoids' characterisation and quantification. Eventually, we present the findings of our current work in an effort to differentiate amongst AI and MA constituents for pesticidal and pharmaceutical activity, and to develop purification techniques as well as identification and quantification analysis methods for the active principal compounds.

Purification and Identification Analysis of Limonoids

Although one third of the limonoids known to date are meliacins, the chronology of the evolution of qualitative and quantitative analysis is devoted to limonoids contained in Rutaceae. This is not only because of their contribution towards the development of pharmaceuticals and pest management, but also because they have been greatly studied in relation to their nutritional properties and contribution to human health (*47*). The interest in studying citrus limonoids has been greater since some of them (bitter aglycones) are responsible for

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producing bitterness in citrus fruits. The need to monitor delayed bitterness in citrus fruit, usually caused by physical damage or a field freeze event, has spawned a wide variety of analytical methods to quantify aglycones, to remove or mitigate the bitterness of fruits and juice (*2*). Amongst isolation methods from citrus fruit are solvent extraction, buffer extraction and supercritical fluid methods, while further purification is usually done by means of fractional crystallization or chromatography (*48*). The milestones in the chemistry of limonoids research have been summarised by Patil and co-workers in 2009 (*49*). Briefly, limonin [2] was the first ever isolated limonoid, extracted from navel orange juice in 1938 and its structure was established 42 years later by means of X-ray crystallographic techniques. The first application of Nuclear Magnetic Resonance (NMR) spectroscopy on limonoids took place in 1960, while thin layer chromatography (TLC) and High Pressure Liquid Chromatography (HPLC) techniques were developed in 1970 and 1975 respectively. Limonoids detection on TLC is usually performed using the Ehrlich's reagent (*50*). The quantification of limonoids by means of HPLC has improved since its inception by including a higher number of compounds in normal and reversed phase. The HPLC-UV methods require high purity solvents with minimal UV end absorption to accommodate the 207 nm UV absorbance maxima of limonoids. The columns employed in HPLC-UV are usually C-18 bonded silica absorption media and the solvents acetonitrile/water or acetonitrile/aqueous acid mobile phases. These HPLC-UV methods have detection limits near 1 ng. With the advent of liquid chromatography coupled with mass spectroscopy (LC-MS) in 2000 the resolution and precision of HPLC has improved much. Atmospheric Pressure Chemical Ionisation (APCI) and Electrospray Ionization (ESI) LC-MS utilizing reverse phase HPLC systems have shown to be capable of detecting and quantifying at nanogram levels. Nonetheless, the accuracy in the quantification of limonoids by means of LC-MS requires the use of standards, the lack of availability of which severely inhibits the application of HPLC-UV methods to identify and quantify limonoids. Nevertheless, fragmentation patterns obtained by APCI and ESI LC-MS analysis on pure limonoids, can be used as an identification tool for unknown molecules. In addition, ammonia or acetic acid enhanced LC-MS analysis performed in negative or positive ion mode collision can establish specific ion-adduct patterns useful in the identification of limonoids. The characteristic fragment ions and fragmentation patterns obtained by mass spectrometry analysis make it an important tool in limonoids characterisation even when molecular ions or protonated molecular ions are observed at low abundance or are not discernable, since it can be used as a reference to confirm function groups in molecules' structure (*48*, *51–55*). Other identification techniques used for limonoids identification are the High Resolution Fast Atom Bombardment analysis (HR-FAB-mass spectrum), the Infrared spectroscopy (IR) analysis and the Electron Impact Mass Spectrometry (EIMS) (*56–58*).

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Purification and Chemical Characterisation of the Limonoids Isolated from AI and MA

Meliacins are usually purified from the organic fractions (CH_2Cl_2, EtAOc or ether) of polar crude extracts (MeOH or EtOH) obtained from various parts of MA. The chromatographic separation and fractions' purification is performed on open column chromatography (CC) packed with silica gel or Sephadex, flash (FC) and Medium Pressure Chromatography (MPLC), as well as by normal or reversed column (semi-) preparative HPLC. Fractions' monitoring is usually performed by TLC observation under UV_{254} nm or by spraying plates with anisaldehyde or Ehrlich's reagent. The mass spectra analysis is performed on EI-MS, FAB-MS and Secondary Ion Mass Spectrometry (SIMS), while structure delineation is achieved by means of IR spectroscopy as well as ^{13}C and ^{1}H NMR. Optical rotation is usually measured as well (*20*, *59–80*). Seldom has GC-MS analysis coupled with mass spectra libraries (NIST) been involved in MA components identification like in the case of the cumarine scopoletin as well as volatile compounds present in flowers (*81*, *82*), while amongst more recent mass spectrometry techniques is the matrix-assisted laser desorption/ionization (MALDI) (*7*). Interestingly, in the case of *Melia toosendan* a High Resolution Time of flight Electrospray Ionisation Mass Spectrometer (HR-TOF-ESI MS) together with a 2D-NMR were employed for identification of 12-*O*-ethyl-1-deacetylnimbolinin B [3] and 1-tigloyl-1-*O*-debenzoylohchinal [4] (*83*). In addition, low resolution mass spectra analysis ESI-LC was deducted both in positive and negative ionization in *M. toosendan* extract for the identification and quantitation of toosendanin; to date this is the only commercial Meliaceae limonoid apart from azadirachtin (*84*).

Chemical Characterisation of Cytotoxic and Antiproliferative Limonoids as Well as Nematicidal Aldehydes, Alcohols, and Carboxylic Acids in AI and MA

Since AI-based insecticides are widely used in agriculture we used five methanolic seed extracts of AI together with a commercial formulation to determine insecticidal azadirachtoids content by liquid chromatography/mass spectrometry (LC/MS). According to our results on average, seed extracts contain azadirachtin A (10.9%) [1], azadirachtin B (3.5%) [5], nimbin (10.4%) [6], and large quantities of salannin (19.0%) [7]. We concluded that the composition of commercial formulations may present different azadirachtoids content depending on the natural extracts used in the preparation, this may explain differences in field efficacy for these formulations (*38*). We further developed a rapid and accurate identification and quantification method of the azadirachtoids as well as their deacetylated analogues on tomatoes and peaches. Using a HPLC/ESI tandem mass spectrometer, azadirachtoids were selectively detected monitoring the multiple reaction transitions of sodium adduct precursor ions (Figure 1) and the presence of interfering compounds was found to be minimal (*85*).

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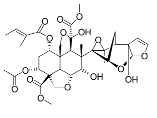
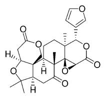
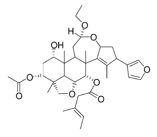
Table 1. Limonoids isolated from Melia azedarach (see Appendix B for larger version of table)



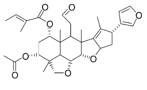
[1] azadirachtin A



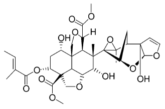
[2] limonin



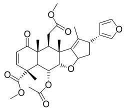
[3] 12-O-ethyl-1-deacetylnimbolinin B
Xie F. et al., 2008



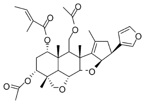
[4] 1-tigloyl-1-O-debenzoylohchinal
Xie F. et al., 2008



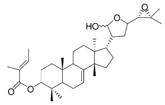
[5] azadirachtin B
Caboni P. et al., 2006



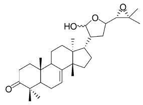
[6] nimbin
Caboni P. et al., 2006



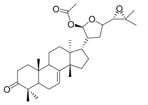
[7] salannin
Caboni P. et al., 2006, Huang et al1994



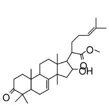
[8] 3-α-O-tigloylmelianol
Ntalli N. et al. 2010



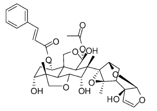
[9] melianone
Ntalli N. et al. 2010



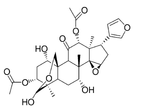
[10] 21-β-acetoxymelianone
Ntalli N. et al. 2010



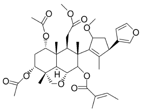
[11] methyl-kulonate
Chiang and Chang, 1973; Huang, 1999



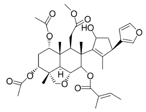
[12] 1-cinnamoyl-3,11-dihydroxymeliacarpinin
Alche et al., 2002



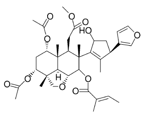
[13] 28-deacetylsendanin
Kim, M. et al. 1999



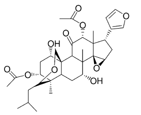
[14] 15-O-deactyl-15-O-methylnimbolidin A
Zhou H., et al. 2005



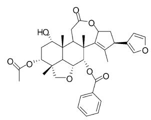
[15] 15-O-deacetyl-15-O-methylnimbolidin B
Zhou H., et al. 2005



[16] 15-O-deacetyl-nimbolidin B
Zhou H., et al. 2005



[17] 12-O-deacetyltrichilin H
Zhou H., et al. 2005



[18] 1-O-deacetylohchinolide A
Zhou et al., 2004

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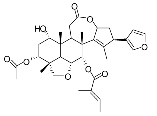
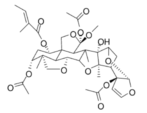
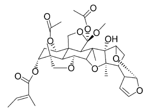
Table 1. (Continued). Limonoids isolated from Melia azedarach (see Appendix B for larger version of table)



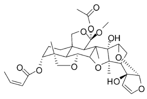
[18] 1-O-deacetylohchinolide B
Zhou et al., 2004



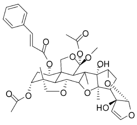
[19] 1-tygloyl-3,20-diacetyl-11-methoxymeliacarpinin
Takeya et al., 1996 [a]



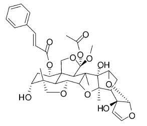
[20] 3-tigloyl-1,20-acetyl-11-methoxymeliacarpinin
Takeya et al., 1996 [a]



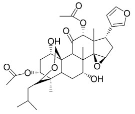
[21] 1-deoxyl-3-methacrylyl-11-methoxymeliacarpinin
Takeya et al., 1996 [a]



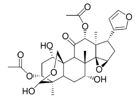
[22] 1-cinnamoyl-3-acetyl-11-methoxymeliacarpinin
Takeya et al., 1996 [a]



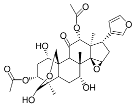
[23] 1-cinnamoyl-3-hydroxyl-11-methoxymeliacarpinin
Takeya et al., 1996 [a]



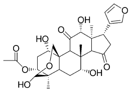
[24] 29-isobutylsendanin
Kipassa N., 2008



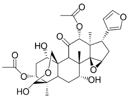
[25] 12-hydroxyamoorastatin
Nakatani et al., 1998, Carpinella et al., 2003,



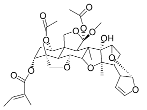
[26] 29-deacetylsendanin or toosendanin
Kipassa N., 2008



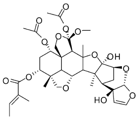
[27] 12-hydroxyamoorastatin
Kipassa N., 2008



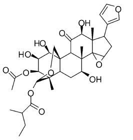
[28] 12-acetoxyamoorastatin
Kipassa N., 2008



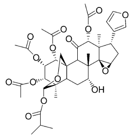
[29] 1-tygloyl-3-acetyl-1-methoxymeliacarpinin
Itokawa H., 1995



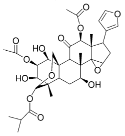
[30] 1-acetyl-3-tigloyl-11-methoxymeliacarpinin
Itokawa H., 1995



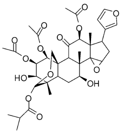
[31] 12-deacetyltrichilin I
Takeya et al., 1996 [b]



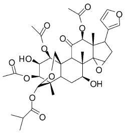
[32] 1-acetyltrichilin I
Takeya et al., 1996 [b]



[33] 3-deacetyltrichilin H
Takeya et al., 1996 [b]



[34] 1-acetyl-3-deacetyltrichilin H
Takeya et al., 1996 [b]



[35] 1-acetyl-2-deacetyltrichilin H
Takeya et al., 1996 [b]

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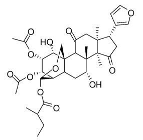
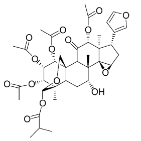
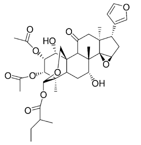
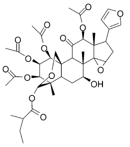
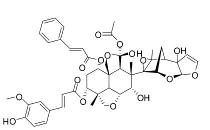
Table 1. (Continued). Limonoids isolated from Melia azedarach (see Appendix B for larger version of table)

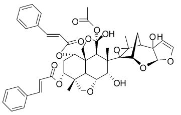

[36] meliatoxin B1
Huang et al., 1994; Takeya et al., 1996

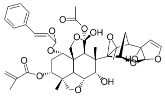

[37] trichilin H
Huang et al., 1994; Takeya et al., 1996 [b]

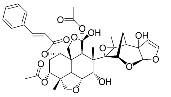

[38] trichilin D
Takeya et al., 1996 [b]

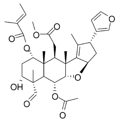

[39] 1,12-di-O-acetyltrichilin B
Takeya et al., 1996 [b]

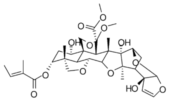

[40] 11-α-hydroxy-1-cinnamoyl-3-feruloyl-meliacarpin
Bohnenstengel et al., 1999; Ayyad et al, 2008

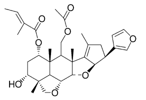

[41] 1,3-dicinnamoyl-11-hydroxymeliacarpin
Bohnenstengel et al., 1999

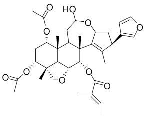

[42] 1-cinnamoyl-3-methacrylyl-11-hydroxy-meliacarpin
Bohnenstengel et al., 1999

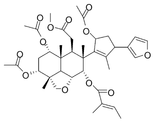

[43] 1-cinnamoyl-3-acetyl-11-hydroxymeliacarpin
Bohnenstengel et al., 1999

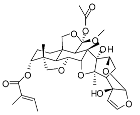

[44] salannal
Huang et al., 1996

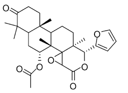

[45] meliacarpinin E
Huang et al., 1996

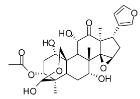

[46] deacetylsalannin
Huang et al., 1996

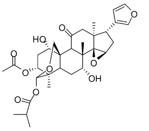

[47] nimbolinin B
Huang et al., 1996


[48] nimbolidin B
Huang et al., 1996


[49] 1-deoxy-3-tigloyl-11-methoxymeliacarpinin
Kipassa N., 2008, Nakatani et al., 1995; Huang et al., 1994


[50] gedunin
Huang, 1999Kipassa N., 2008


[51] meliartenin
Carpinella et al., 2002, 2003


[52] azedarachin C
Huang et al., 1995

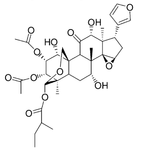

[53] trichilin B
Huang et al., 1994

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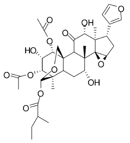
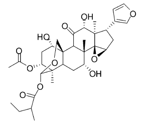
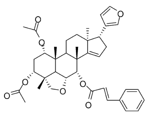
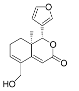
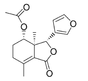
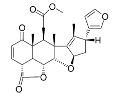
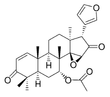
Table 1. (Continued). Limonoids isolated from Melia azedarach (see Appendix B for larger version of table)

 **[55]** azedarachin A *Huang et al., 1994*	 **[56]** nimbolin A *Huang et al., 1994*	 **[57]** nimbolin A *Huang et al., 1994*
 **[57]** azedaralide Nakatani et al., 1998; Okamura et al., 1997 Fukuyama et al., 2006	 **[58]** 12α-acetoxyfraxinellone Nakatani et al., 1998; Okamura et al., 1997 Fukuyama et al., 2006	**[59]** fraxinellone Nakatani et al., 1998; Okamura et al., 1997 Fukuyama et al., 2006
 **[60]** nimbolide *Kipassa N., 2008*	 **[61]** epoxyazadiradione *Kipassa N., 2008*	

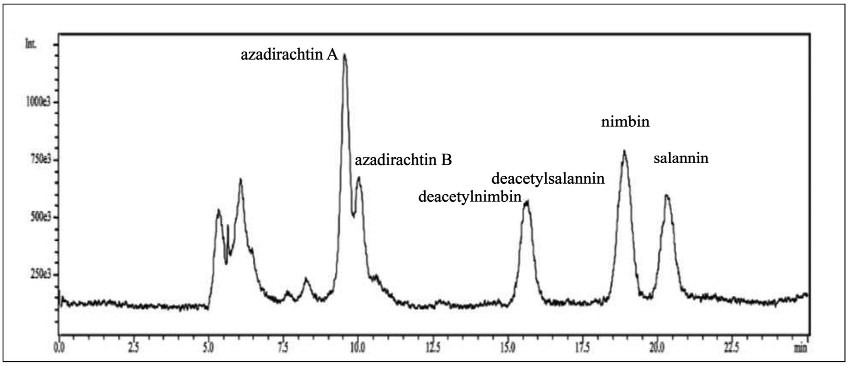


Figure 1. Chromatogram of the methanol extract of neem fruits in ESI$^+$ LC-MS.

We also made a phytochemical investigation on the limonoids rich dichloromethane-soluble part of the methanol extract obtained from the fruits of MA. In particular, we separated and purified the limonoids by using successive open column chromatography on silica gel and then determined their structure mainly by 1D and 2D NMR experiments as well as HPLC-Q-TOF mass spectrometry. According to our results MA afforded one new tirucallane type triterpene, 3-*α*-O-tigloylmelianol [8] and three known tirucallanes, melianone [9], 21-*β*-acetoxy-melianone [10] and methyl kulonate [11] (Figure 2). We proved that 21-β-acetoxy-melianone [10], 3-α-O-tigloylmelianol [8] and melianone [9] were cytotoxic while 21-β-acetoxymelianone [10], and 3-α-O-tigloylmelianol [8] showed an additional moderate antiproliferative effect on the human lung

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adenocarcinoma epithelial cell line A549 (*86*) (Figure 3). Also in our previous studies we had proved that other limonoids isolated from extracts of MA such as meliacin exhibit potent antiviral activity against herpes simplex virus type 1 (HSV-1) (*87–90*). Likewise, 1-cinnamoyl-3,11-dihydroxymeliacarpin [12], reduces both, *Vesicular stomatitis* virus (VSV) and *Herpes simplex* virus type 1 (HSV-1) multiplication (*70*, *91*, *92*), it impedes nuclear factor kappaB (NF-kappaB) activation in HSV-1 infected conjunctive cells (*93*) and it interferes with the normal transport of cellular glycoproteins (*23*).

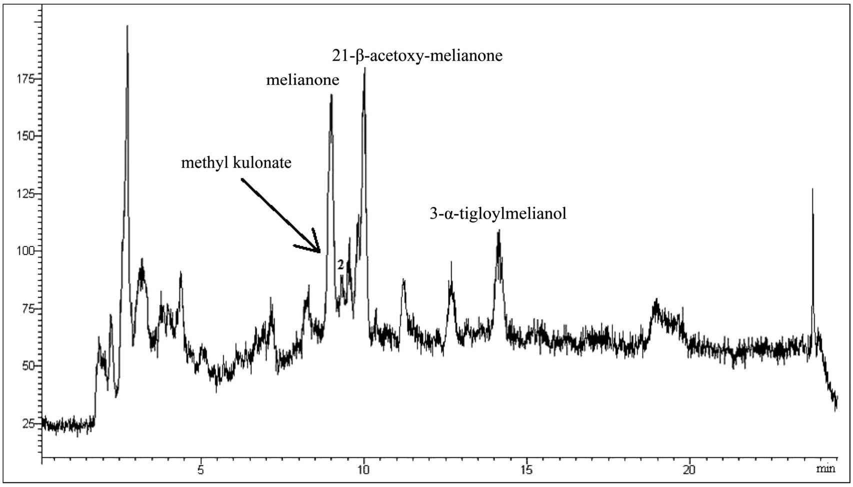

Figure 2. Chromatogram of the dichloromethane part of the methanol extract of chinaberry fruits in ESI$^+$ LC-MS.

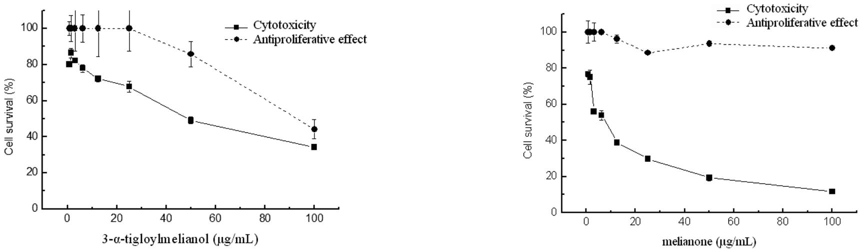

Figure 3. Regression curves of cytotoxic and antiproliferative activity of 3-α-tigloylmelianol and melianone against the tumorigenic cell line A549.

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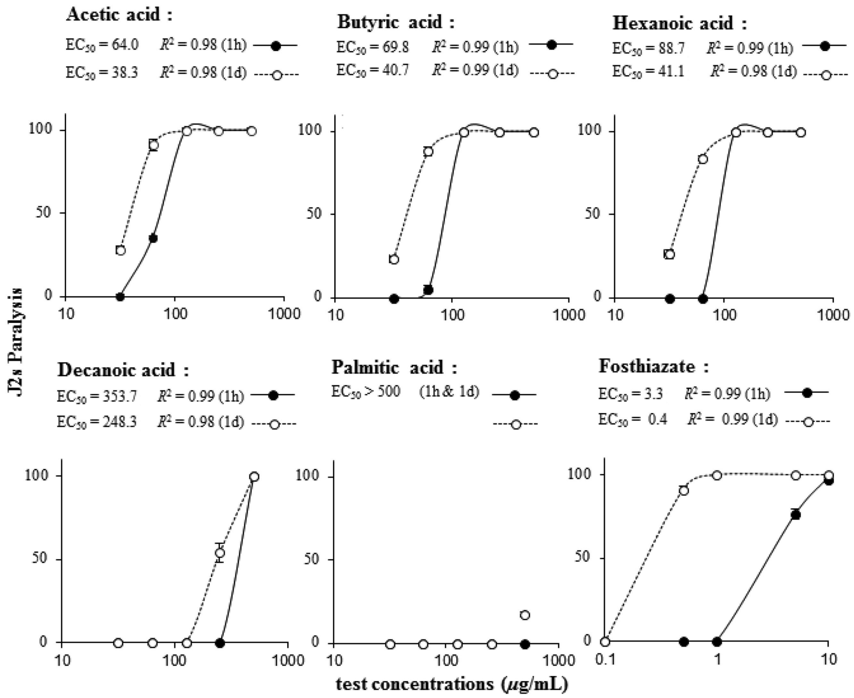

Figure 4. Regression curves of M. incognita paralysis following J2 immersion in solutions of pure organic acids and fosthiazate for 1h and 1d. Each point represents the average % number of paralysed J2 of six replications per treatment (two experiments replications) after elimination of natural paralysis / death observed in the control. Standard deviations of mean data values are presented as error bars.

Focusing on the pesticidal properties of limonoids, we proved that a commercial azadirachtin nematicide (Neemazal® 1%EC, Intrachem Hellas) fails to protect crops from Meloidogyne incognita infection and that it exhibits only fair activity at concentrations way higher than the registered concerning nematodes' paralysis and biological cycle arrest (12.8 mg a.i. L^{-1} and 30.72 μg a.i. g^{-1}) (*94*). On the contrary, the Greek MA pulverised fruits were proved to possess strong nematicidal properties when incorporated in *M. incognita* infested soil and to arrest nematode life cycle (EC_{50} = 0.34 % w/w). By performing paralysis activity bioassays we proved that the nematicidal activity of MA fruits lays on the polar compounds present in its defatted methanol fruit extract ($EC_{50/96h}$ = 0.0426 %) that was also found to arrest nematodes life cycle at 50 % when incorporated in soil at the concentration of 0.916 % w/w (*25*). Most interestingly, we proved that the limonoids contained in MA, as reported above, had no effect on nematodes when tested at up to 500 μg/mL suggesting that the nematicidal activity lays elsewhere. Moreover, we used the nematicidal defatted methanol extract of the fruits of MA to separate polar components on a fused silica capillary Varian CP-WAX 57CB and identify them by comparison of their relative retention times and mass fragmentation with those of authentic standards and computer matching against the NIST98 library. Amongst the components we identified organic acids, aldehydes and alcohols, which were thereafter tested for nematode paralysis activity. According to our results strong dose and time response relationships were established concerning nematodes paralysis for all substances. The organic acids were found to have a nematicidal effect at the dose range of 31.2-500 μg/mL in the decreasing order of acetic ($EC_{50/1\,h}$ = 64.0 μg/mL), butyric ($EC_{50/1\,h}$ = 69.8 μg/mL), hexanoic ($EC_{50/1\,h}$ = 88.7 μg/mL), and decanoic ($EC_{50/1\,h}$=353.7 μg/mL), revealing the linear relationship of C atom number and nematicidal activity (Figure 4). The aldehyde furfurale was the foremost nematicidal compound, exhibiting activity similar to that of the commercial nematicide fosthiazate, both after J2 immersion in test solutions and after exposure to its vapours (fumigant activity) (Figure 5). This nematicidal-biofumigant toxicity of MA is a rather important property enhancing its activity to the non-treated soil layers where nematodes are usually protected and it reveals this species' potency of incorporation into integrated pest management program (IPM) (*95*).

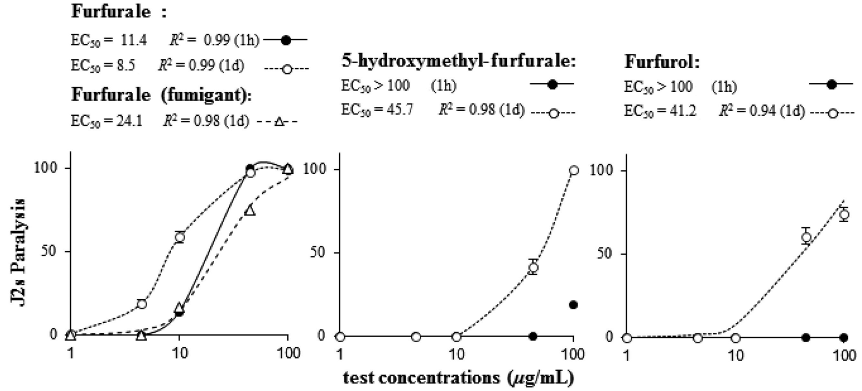

Figure 5. Regression curves of M. incognita paralysis following J2 immersion in solutions of aldehydes and alcohols for 1h and 1d. Concerning furfurale, data are presented after nematodes immersion in test solutions and exposure to its vapours. Each point represents the average % number of paralysed J2 of six replications per treatment (two experiments replications) after elimination of natural paralysis / death observed in the control. Standard deviations of mean data values are presented as error bars.

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Biological Activities of MA

Pharmaceutical and Veterinary Uses

The pharmaceutical activity of MA has been greatly studied given that MA has participated in traditional Indian medicine for thousands of years. Some of the main activities proven in the recent years are presented herein. Crude leaf extracts of MA exerted an antiviral action on the development of herpetic stromal keratitis when supplied by post treatment while treatment 96 h post infection prevented ocular disease suggesting an *in vivo* immunomodulating activity (*90*). Also 28-deacetylsendanin [13], a compound purified from fruits of MA, exerted antiviral activity on herpes simplex virus-1 on Vero cells in a dose dependent manner. The IC_{50} value calculated was 1.46 µg/mL without cytotoxicity at 400 µg/mL. The 28-deacetylsendanin [13] inhibited the replication of HSV-1, reduced the synthesis of HSV-1 thymidine kinase and led to the formation of defective nucleocapsids (*96*). Meliacine was found to affect two events of the Junin virus replicative cycle in Vero cells that require membrane fusion: uncoating and budding. In particular it was found to block virus penetration by preventing the uncoating step, it interfered with the release of infectious particles to the extracellular medium and inhibited the low-pH-induced fusion of infected cells (*97*). Four C-seco-limonoids and a tetracyclic one; namely, 15-*O*-deacetyl-15-*O*-methylnimbolidin A [14] and B [15], 15-*O*-deacetylnimbolidin B [16] and 12-*O*-deacetyltrichilin H [17], isolated from the methanol extract of the ripe fruits of MA, were found to exhibit significant antiproliferative activity on Hela S3 cell line (human epithelial cancer) and the IC_{50} values were calculated at 0.1 to 37.4 µM (*62*). Also 1-*O*-deacetylohchinolide A [18] and 1-*O*-deacetylohchinolide B isolated from the methanol extract of the ripe fruits of MA exhibited significant inhibitory activity on Hela S3 cells and the IC_{50} values were calculated at 2.4 and 0.1 µM, respectively (*75*). A series of methoxymeliacarpins derivatives isolated from MA were found active on P388 lymphatic leukemia cells. These are 1-tigloyl-3,20-diacetyl-11-methoxymeliacarpinin [19], 3-tigloyl-1,20-diacetyl-11-methoxymeliacarpinin [20], 1-deoxyl-3-methacrylyl-11-methoxymeliacarpinin [21], 1-cinnamoyl-3-acetyl-11-methoxymeliacarpinin [22] and 1-cinnamoyl-3-hydroxyl-11-methoxymeliacarpinin [23]. The IC_{50} values ranged from 1.5 to 100 µg/mL (*71*). 29-isobutylsendanin [24], 12-hydroxyamoorastatin [25], and 29-deacetylsendanin [26] also exhibited strong cytotoxic activity on P388 lymphatic leukemia cells with IC_{50} values of 0.034, 0.090 and 0.026 µg/mL respectively. Finally 12-hydroxyamoorastatone [27], 12-hydroxyamoorastatin [25] and 12-acetoxyamoorastatin [28] were found significantly cytotoxic on A-549 (human lung adenocarcinoma), SK-OV-3 (human ovarian adenocarcinoma), SK-MEL-2 (human malignant melanoma), XF-498 (human CNS carcinoma) and HCT-15 (human colon adenocarcinoma), 12-acetoxyamoorastatin being the most active one (ED_{50}=0.0007 to 0.04 µg/mL) (*98*). A new heteropolysaccharide purified from MA fruit exhibited cytotoxic activity on BGC-823 cell line (*99*). Itokawa and co-workers proved the cytotoxic activity on P388 cell line *in vitro* (IC_{50} = 1.3 to 7.9 µg/mL) for two new azadirachtin-type limonoids (1-tigloyl-3-acetyl-11-methoxymeliacarpinin

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[29] and 1-acetyl-3-tigloyl-11-methoxymeliacarpinin [30]) together with three sendanin-type limonoids (29-isobutylsendanin [24], 12-hydroxyamoorastin [25], and 29-deacetylsendanin [26]) and their acetylated derivatives (*73*). Takeya and co-workers have proven the cytotoxic activity of five trichilin-type limonoids, extracted from ethanolic MA root bark extract against P388 cell line *in vitro*. These were 12-deacetyltrichilin I [31], 1-acetyltrichilin H [32], 3-deacetyltrichilin H [33], 1-acetyl-3-deacetyltrichilin H [34], 1-acetyl-2-deacetyltrichilin H [35], meliatoxin B_1 [36], trichilin H [37], trichilin D [38] and 1,12-diacetyltrichilin B [39]. The IC_{50} values varied from 0.011 to 5.4 μg/mL (*69*). A meliacarpin derivative, 11-α-hydroxy-1-cinnamoyl-3-feruloyl-meliacarpin [40] has proven cytotoxic on a human tumorogenic cell line MCF-7 of human breast carcinoma (LC_{50}=1.75 μM) (*60*). *Melia azedarach* drupe ethanol extract was found to possess anthelmintic activity against the pork tapeworm *Taenia solium* (Cestoda: Taeniidae), the sheep hookworm *Bunostomum trigonocephalum* (Nematoda: Ancylostomatidae), the nodular worm *Oesophagostomum columbianun* (Nematoda: Chabertiidae) and the earthworm *Pheretima posthuma* (Annelida: Megascolecidae) at a concentration of 0.2% (*18*). Recently, Kamaraj and co-workers have reported the anthelmintic action of aqueous as well as hydro-alcoholic extracts of leaves and seed of MA on *Haemonchus contortus* (Stronylida) (*100*). Madibela and Kelemogile have proved that MA extracts act as natural control agents of coccidiosis and more specifically on *Eimeria* spp (*17*). Moreover, besides acting on insects pests MA extracts also act on insect parasites or vectors of diseases. Valladares and co-workers have demonstrated that instar nymphs of *Triatoma infestans* (Hemiptera: Reduviidae), a vector of Chagas disease, when reared in contact with MA extract-treated refuges were significantly smaller than controls after molting. The ethanol extract of unripe fruits was found more active than ripe fruits' extract, while leaves were found ineffective (*101*). Hexane and $CHCl_3$ extract of MA ripe fruits inhibited egg production and embryogenesis of *Boophilus microplus* (Acari: Ixodidae), a cattle parasite. The mortality was concentration and time dependent and higher values were observed at concentrations of 0.125 and 0.25%. The activity obtained from apolar and intermediate polarity solvents can be attributed to components structurally related to steroids and terpenoids (*102*). Phagoinhibitor and anti-molting activity were observed in four lignanes of MA namely pinoresinol, bis-epi-pinoresinol, hemicetal and diacid against *Rhodnius prolixus* (Hemiptera: Reduviidae), a vector of Chagas disease (*103*, *104*). A senescent leaf extract of MA was proved to possess highly effective larvicidal, growth regulating and oviposition deterrent activity against *Aedes aegypti* (Diptera: Culicidae) (*26*). Strong larvicidal, pupicidal, adulticidal, antiovipositional activity, repellency and biting deterrency was observed after exposure to methanolic extracts of seeds and leaves from MA at the concentration of 2% (*105*) on *Anopheles stephensi* (Diptera: Culicidae), a malaria vector. An ethanol extract of MA, containing flavonoids, lignans and triterpenes, exhibited ovicidal and pediculicidal activity on the head louse *Pediculus humanus capitis* (Phthiraptera: Pediculidae) (*106*). The essential oil extracted from flowers of MA, possessing small molecular aliphatic compounds, was found to possess antibacterial activity (*82*) while in the past such activity has also been attributed to MA limonoids' contents (*80*). Aqueous and alcoholic

extracts of MA leaves have been found to possess significant antioxidant activity in comparison to AI. The high scavenging property may be due to the hydroxyl groups existing in the phenolic compounds chemical structure (*107*). Recent reviews on MA pharmaceutical properties delineate activities such as antitumoral, antiviral, antifungal, analgesic, haematological, immunomodulatory, antiviral, antibacterial, cytotoxic, antimalarial, anthelmintic, antilithic and antifertility (*5*, *108*). Toxicity issues have stopped MA from being widely known like AI since some compounds contained in its extracts, like meliatoxins, are considered toxic to mammals (*109*). However, MA fruit extracts' oral toxicity to rats was calculated above 16 g/kg (*110*).

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Pesticidal Uses

The insecticidal activity of various Meliaceae species such us MA have long been demonstrated (*42*). It is a deciduous tree that is native to north-eastern India. It has several common names such as, White cedar, Persian lilac, Tulip cedar and chinaberry. It has been introduced into several countries in Asia, North and Latin America as well as Europe. Extracts of its fruits, seeds and leaves have shown many properties amongst which pesticidal activity (*108*). Diet treatment with methanolic extracts of leaves and seeds of MA tree were found to inhibit the growth, feeding and oviposition of *Hyblaea puera* (Lepidoptera: Hyblaeidae), an insect that defoliates teak tree. Larval and pupal duration was extended, growth regulatory effects were observed, such as larval-pupal intermediates with deformed wings and abdomen, while adult longevity and fecundity were reduced. The seed extracts were found more active that leaf extracts, with the highest dose of 4% seed extract providing 94% feeding deterrence. Food consumption, digestion, relative consumption rate (RCR), efficiency of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD), and relative growth rate (RGR) values declined significantly. Larvae chronically exposed to MA extract showed a reduction in weight of 65-84% (*111*). Senthil Nathan in 2006 observed similar antifeedant effects of the methanolic seed extract of MA on the rice leafhopper *Cnaphalocrosis medinalis* (Lepidoptera: Pyralidae). In particular at the concentration of 2% the fourth instar larvae suffered a reduction of acid and alkaline phosphatases, adenosine triphosphatases as well as lactate dehydrogenase activities (69%, 71%, 46 % and 52 % respectively) (*112*). Unripe fruits as well as green and senescent leaves of MA at 1-10 % significantly deterred feeding by the adults of the elm beetle *Xanthogaleruca luteola* (Coleoptera: Chrysomelidae) and the subsequent starvation interfered with the mortality values observed (*113*). Akhtar and co-workers have reviewed on the biological activities of various Meliaceae species on *Trichoplusia ni* and *Pseudaletia unipuncta* (Lepidoptera: Noctuidae) and have concluded that an interspecific variability in susceptibility exists, with *T. ni* being the most susceptible species. In specific the refined bark extract of MA (3% toosendanin [26]) was found to exhibit respectively against the two species: growth inhibition (EC_{50}=100 and 2.26*10^4 μg/mL), toxicity through feeding (EC_{50}=6.01*10^4 and 3.06*10^4 μg/mL), toxicity through spraying (EC_{50}=1.32*10^5 and 1.26*10^5 μg/mL) and feeding deterrence (EC_{50}=288 and 248.9 μg/cm^2) (*36*). The ethyl-ether fraction of the methanol

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extract obtained from MA fruits was found to have a "reduced fitness" effect on *Spodoptera frugiperda* (Lepidoptera: Noctuidae). When tested on artificial diet at 0.01%; it inhibited 31% the cholinesterase activity in muscular contraction and it additionally induced a 34% activation of the NADPH-cytochrome *c* (P-450) reductase activity. Cytochrome *c* (P-450) enzymes participate in the detoxification mechanism of the insect and facilitate its adaptation to physiologically new conditions by degrading lipophilic xenobiotics, such as flavonoids and terpenoids (*114*). Leys and co-workers distinguished amongst MA methanol extract's fractions for growth disrupting activity on *S. frugiperda* and proved that the ethyl acetate exhibited the highest one (*115*). Survival, fecundity, development, oviposition and feeding disruptive activities, of MA leaf aqueous extracts on the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae), have been demonstrated in laboratory and verified in field experiments. Interestingly no effects were observed on the parasitoids *Cotesia plutellae* and *Diandromus collaris* contributing to final crop (cabbage) protection (*116*). Also Charleston and co-workers proved under laboratory and glasshouse conditions the absence of direct negative effects of MA extracts on longevity of the diamondback moth parasitoids *Cotesia plutellae* and *Diadromus collaris* (*117*). Adults' deformation, pupal reduced weight and viability as well as ovicidal action were observed on *P. xylostella* after exposure to aqueous extracts of MA (*118*). The methanolic extract (10%) of the stems of MA exhibited ovicidal activity against *Helicoverpa armigera* (Lepidoptera: Noctuidae); while the petroleum ether and acetone fruit extracts had antifeedant activity against *Sitophilus oryzae* (Coleoptera: Curculionidae) (*5*). Aqueous extracts of leaves and fruits of MA significantly lowered the number of *Liriomyza huidobrensis* (Diptera: Agromyzidae) larvae under field conditions and performed comparable efficacy to the abamectin, cyromazine, imidachloprid, pyrazophos and azadirachtin. Interestingly, MA treated larvae were similarly deformed to those treated with cyromazine; that is they were partially brown, rotting and oozing. On the contrary imidachloprid treated larvae were dry, non-intact and dark in colour. This implies that MA may have a mode of action similar to that of the triazine insect growth regulator cyromazine (*119*). Feeding deterrence as well as oviposition rates reduction activities of MA against *L. huidobrensis* were observed by Bancio and co-workers who additionally proved translaminar action of the extracts on *Vicia faba* plants (*120*). Aqueous, methanol, ethanol and ether extracts of MA fruits were found to possess repellent activity on the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) and the most significant activity was shown by the methanol extract. In addition host preference experiments have evidenced that MA is not a good host for sweetpotato whitefly (*121*). Oviposition and adult emergence inhibition activities of extracts of callus, fruits and leaves of MA were studied against *B. tabaci*. Results showed that aqueous and methanolic extracts decreased similarly the oviposition rate. Additionally, plant parts' storage in -20°C for one year prior to extraction did not seem to interfere with the activity, this indicates the probable stability of active compounds under reported conditions (*122*). The methanolic extract of MA leaves yielded 1,3-dicinnamoyl-11-hydroxymeliacarpin [41], 1-cinnamoyl-3-methacrylyl-11-hydroxymeliacarpin [42] and 1-cinnamoyl-3-acetyl-11-hydroxymeliacarpin [43] which were confirmed by artificial diet

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experiments as its principal insecticidal and growth disrupting constituents on *Spodoptera littoralis* (Lepidoptera: Noctuidae). The EC_{50} values were calculated at 0.57, 0.57 and 0.27 μg/mL respectively (Figure 1) The LC_{50} value calculated for 1-cinnamoyl-3-methacrylyl-11-hydroxymeliacarpin [42] on *Spodoptera littoralis* was 0.48 μg/mL this makes its activity similar to that of azadirachtin (*59*). Schmidt and co-workers studied the neuroendocrine effect of the methanolic MA fruit extract on *S. littoralis* and *Agrotis ipsilon* (Lepidoptera: Noctuideae) by performing artificial diet bioassays. They concluded that the treated larvae had significantly reduced corpora allata (CA) volume, increased juvenile hormone (JH) levels in hemolymph and significantly decreased hemolymph protein content, this was accompanied by morphology and physiology changes of the gut (*123*). Antifeedant properties on *Spodoptera eridania* (Lepidoptera: Noctuidae) were also exhibited by the six seco limonoids isolated from root bark diethyl ether extract of MA, namely: salannal [44], meliacarpinin E [45], salannin [7], deacetylsalannin [46], nimbolinin B [47] and nimbolidin B [48]. The most potent antifeedant was meliacarpin E [45] at 50 μg/mL, while other C-seco limonoids were active at 500 to 1000 μg/mL (*63*). A limonoid isolated from the MA root bark, 1-deoxy-3-tigloyl-11-methoxymeliacarpinin [49], was found to induce complete inhibition of feeding of the larvae of *Spodoptera exigua* at 3 μg/cm^2. Three meliacarpins derivatives, 1,3-dicinnamoyl-11-hydroxymeliacarpin [41], 1-cinnamoyl-3-methacrylyl-11-hydroxymeliacarpin [42] and 1-cinnamoyl-3-acetyl-11-hydroxymeliacarpin [43] exhibited EC_{50} values of 0.57, 0.57 and 0.48 μg/mL respectively towards the larvae of *S. littoralis*. Gedunin [50], photogedunin epimeric mixtures (1 + 2) and photogedunin acetates (3+4) showed high larval growth reduction activities at 50 to 52 μg/mL (*98*). Systemic fractionation of the ethanol extract of MA kernels led to the isolation of a mixture of interchangeable isomers, namely meliartenin [51] and 12-hydroxyamoorastatin [25], exhibiting antifeedant activity on *Epilachna paenulata* (Coleoptera: Coccinelidea) and *S. eridania* (4 μg/cm^2 and 1 μg/cm^2, respectively), higher than that achieved by toosendanin (*72*). When the interchangeable isomer 12-hydroxyamoorastatin [25] was tested individually its ED_{50} value, calculated at 0.80 μg/cm^2, was comparable to that of azadirachtin A [1] and lower than that of toosendanin [26] (0.72 and 3.69 μg/cm^2, respectively). In no choice tests, *E. paenulata* larvae reared on food treated with meliartenin [51] and 12-hydroxyamoorastatin [25], ate less, gained less weight and suffered greater mortality than control larvae. The activity of the two interchangeable isomers was comparable and the LD_{50} values were calculated at 0.76 and 1.24 μg/cm^2, respectively (*74*). Azedarachin C [52], exhibits antifeedant activity at 400 μg/mL (corresponding to a concentration of 8 μg/cm^2) on *S. exigua* (*68*). Strong antifeedant activity on *S. exigua* was also exhibited by fifteen limonoids isolated from the ether extract of the root bark of MA. The meliacarpinins were the most potent since they were found active at 50-100 μg/mL, corresponding to the concentration of 1-2 μg/cm^2. This activity is weaker than azadirachtin's but stronger than the one exhibited by trichilins or azedarachins with a 14,15-epoxide and a 19/29 lactol bridge, in which the 12α-OH compounds, thrichilin B [53], aphanastatin [54] and azedarachin A [55] were most potent and active at 200 μg/mL. Independently of the substitution patterns in ring A and the C-28 ester moieties, the 12-acetoxy and 12-deoxy compounds were active at

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400 µg/mL, while nimbolin A [56] and salannin [7] showed weaker activity at 600 µg/mL (*76*). These findings are in accordance with those of Nakatani and co-workers who showed that the antifeedant activity of the limonoids isolated from the root bark of MA on *S. exigua* and *S. eridua* in a decreasing order were as follows: meliacarpins (50 µg/mL) followed by the 12*α*-OH compounds of trichilins and azedarachins with a 14,15-epoxide and a 19/29 acetal bridge (200 µg/mL), and then 12-deoxy type and 12-acetates (400 µg/mL). Nimbolinin B [47] and salannin [7] were poor antifeedants, active at 1000 µg/mL (*61*). The hexane extract from senescent leaves and the ethanol extract from seed kernels of MA exhibited fungistatic activity on *Aspergillus flavus*, *Diaporthe phaseolorum* var. meridionales, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium verticilioides* and *Sclerotinia sclerotiorum* and the minimum inhibition concentration (MIC) values ranged from 0.5 to 25 mg/mL and 0.5 and 5 mg/mL respectively. Three isolated compounds displaying individual and paired activity on *F. verticilliodes* were vanillin, 4-hydroxy-3-methoxycinnamaldehyde and (±)-pinoresinol (*20*). The fungicidal activity of the dichloromethane fraction of the methanol extract obtained from MA seed kernel on *Fusarium verticillioides* was attributed to its content in scopoletin, vanillin, 4-hydroxy-3-methoxycinnamaldehyde (Figure 1) and pinoresinol. The paired activity of these limonoids was characterised by synergistic potentiation and was found similar to the one observed in standards Mancozeb and Carboxin treatments (*21*). The ethanol extract of MA ripe fruits exhibited fungistatic and fungicidal activities on *Aspergillus flavus* and *Fusarium moniliforme*. The MIC as well as minimum fungicide concentration (MFC) values were calculated at 300 and 70 as well as 500 and 100 mg/mL, respectively (*110*). Ethanol bark and fruit extracts of MA exhibit bacteriostatic activity on *Aspergillus niger* and *Trichoderma viride* and the MIC values were calculated at 0.5 % (*124*). Khan and co-workers proved that the antimicrobial activity of MA methanol bark extract lies in its dichloromethane fraction (*125*). *Melia azedarch* was found to possess significant allelopathic potential against radish in terms of seed germination, shoot and root elongation and dry weight. All parts of plant such as leaves, stem and root possessed allelopathic potential (*126*). Apart for the intact limonoids also degraded ones isolated from MA roots such as azedaralide [57], 12*α*-acetoxyfraxinellone [58] and fraxinellone [59] have been found to exhibit antifeedant activity on *Spodoptera* sp as well as other biological activities (*64*, *65*, *127*). Last, monoterpenes isolated from *Penicillium* sp, an endophytic fungus of the roots of MA, were found to possess bacteriostatic effects (*128–130*)

Biological Activities of AI

Pharmaceutical and Veterinary Uses

Nowadays there is an enormous pressure to explore local botanical resources, handy to combat the human deficits and improve quality of life. One of such available resources with great potentials in the 21st century is AI. Since ancient time it has been extensively used in Ayurveda, Unani and Homeopathic medicines. The importance of AI has been recognized by the US National Academy of Sciences, which published a report in 1992 entitled “Neem-a tree for solving global problems" (*131*). The chemical investigation on the products of AI was extensively undertaken in the middle of the twentieth century. Ever since considerable progress has been achieved regarding the medicinal applications of about 135 different types of compounds found in every part of the tree. These substances have been divided into two major classes: isoprenoids and others. Isoprenoids consist of diterpenoids and triterpenoids such as protomeliacins, limonoids, gedunin [50] and vilasin type compounds as well as C-secomeliacins such as nimbin [6], salannin [7] and azadirachtin A [1]. The nonisoprenoids class includes amino acids, carbohydrates, sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin, tannins and aliphatic compounds (*131*, *132*). The diverse biological applications of AI, based on its numerous bioactive ingredients, could be summarised as: antiallergenic, antidermatic, antifeedent, antiviral, antifungal, anti-inflammatory, antipyorrhoeic, antiscabic, anti-implantation, spermatocidal, antimalarial, antioxidant, anti-diabetic, antileishmanial activity, anti-tuberculosis, hepatoprotective, wound and cut healing as well as activities against psoriasis, acne, *Condylomata acuminate*, gastrointestinal helminthiasis, endo- and ectoparasites and herpes simplex (*133–145*). Gedunin [50], present in AI leaves, is the most potent antimalarial limonoid to be reported (IC_{50} = 20 and 39 ng/mL against chloroquine and chloroquine-resistant clones of *Plasmodium falciparum* respectively). Nimbolide [60], a limonoid isolated from leaves of AI as well, inhibited the growth of *Plasmodium berghei* in vitro with ED_{50} = 2 μM. Nimbolide [60], epoxyazadiradione [61], salannin [7] and nimbin [6] were found to be toxic to mammalian and insect cell cultures (143.TK-osteosarcoma: human, N1E-115 nuroblastoma: mouse, Sf9: insect). The most potent was nimbolide [60] with an IC_{50} averaging 6 μM for the three cell lines. Salannin [7] showed a significant protective activity on aspirin induced lesion at oral doses of 10, 20, 50 mg/kg, fact that demonstrates its anti-ulcer activity (*98*). Finally, Saxena and co-workers have demonstrated that AI seed cake can be a good source of nutrition in animal feed if made palatable for use by detoxification using methanol; that is extracting its limonoids contents (azadirachtin A [1], salannin [7], and other bitter constituents) (*146*).

Pesticidal Uses

Neem has long been studied for its biological properties against a broad range of mites and insects (*147*). It has been found to have growth inhibition, chronic toxicity, and antifeedant levels of activity favourably comparable to botanical products at different times in commercial use such as ryanodine, pyrethrum, rotenone and essential oils of rosemary and clove leaf (*36*). In the past 20 years increasingly stringent regulatory requirements in many jurisdictions have allowed only a handful of botanical products to reach the marketplace in Europe, amongst which azadirachtin. The shortage of the natural resource, the standardization and quality control difficulties have been common barriers to commercialization of other botanicals (*148*), while the availability of competing cost-effective products (newer synthetics, fermentation products, microbial) in the past has contributed to their limited success as well (*4*). Nonetheless, the current regulatory environment

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and public health needs are creating opportunities for using of botanicals in situations where human and animal health, are foremost (*149*). In this frame different parts of AI can be processed for safe and economically cheaper uses in the treatment of various diseases and in agriculture (*150*). *Azadirachta indica* produces a vast array of biologically active compounds that are chemically diverse and structurally complex. More than 140 compounds have been isolated from all the different parts of AI: leaves, flowers, seeds, fruits, roots and bark (*151*). Azadirachtin has been the most studied constituent compound and a major subject of intensive research within the scientific community ever since its isolation from AI in 1968. It is considered a green alternative for synthetic insecticides (*152*). There are now over 1000 publications relating to this natural product which cover all aspects of structural, biological and synthetic studies (*153*). Its synthesis was finalised at the University of Cambridge by Ley and co-workers 22 years post its first isolation from AI (*154*). The mode of action of azadirachtin on insects lies in (i) effects on chemoreceptors resulting in antifeedancy; (ii) effects on ecdysteroid and juvenile hormone titres through a blockage of morphogenetic peptide hormone release (e.g. PTTH; allatotropins) and (iii) direct effects on most other tissues resulting in an overall loss of fitness of the insect (*155*). This mechanism of action provides with avoidance of cross resistance (*156*). Interestingly, from a toxicological point of view AI seems to be rather friendly. According to human and animal studies with oral administration of different AI-based preparations the non-aqueous extracts appear to be the most toxic AI-based products, with an estimated safe dose (ESD) of 0.002 and 12.5 μg/kg bw/day. Less toxic are the unprocessed materials, seed oil and the aqueous extracts (ESD 0.26 and 0.3 mg/kg bw/day, 2 μl/kg bw/day respectively). Most of the pure compounds show a relatively low toxicity (ESD azadirachtin A [1] 15 mg/kg bw/day) (*157*). Owing to their mode of action, AI derivatives could be very suitable for IPM. They are primarily feeding poisons of phytophagous insects and therefore they show a considerable selectivity towards natural enemies, especially parasitoids. They do not disturb ecosystems and consequently will not cause outbreaks of new pests, as long lasting insecticides are apt to do. Underprivileged farmers can cultivate their own AI and produce insecticides while in the industrialized countries AI derivatives may open new ways of environmentally sound pest control. Mixtures with other bio products, such as *Bacillus thuringiensis* based pesticides, can increase azadirachtin efficacy on crops of high quality demands (*158*).

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Chapter 5

Phytochemicals from the Fruit and Foliage of Cranberry (*Vaccinium macrocarpon*) - Potential Benefits for Human Health

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Cranberry fruit and foliage (*Vaccinium macrocarpon*) contains bioactive compounds that have been found to limit microbial infections, oxidative processes, inflammation, and cell proliferation. Some of these secondary metabolites also play a protective role in the plant against pests and fungal pathogens. Bioactive constituents in the plant include flavonol and anthocyanin glycosides, oligomeric proanthocyanidins, substituted hydroxycinnamic acids and derivatives, other organic acids, iridoids, carotenoids, and triterpenoids. Because cranberries have a unique and diverse phytochemical composition, multiple protective mechanisms are possible. Recent studies employing *in vitro* and *in vivo* models of human diseases suggest that cranberry fruit is an excellent source of secondary metabolites that help maintain urinary tract and oral health, and protect against certain cancers and other diseases associated with aging. Similar phenolic secondary metabolites have been identified in cranberry foliage, including derivatives of p-coumaric acid and quercetin glycosides that may serve a dual purpose as antioxidants and as protection against pests. Recent data suggests that constituents of cranberry fruit and foliage can inhibit the growth of human fungal pathogens including *Candida* species.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
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Introduction

The American cranberry (*Vaccinium macrocarpon*) of the family Ericaceae is cultivated widely in Northeastern U.S., Wisconsin, the Pacific Northwest and Atlantic Canada. It is a low-growing, woody perennial vine with short vertical upright branches, 5 to 20 cm in height, bearing small ovate leaves. Cranberries were used by Native Americans to preserve dried meats and to treat wounds. Over the years, the consumption of cranberry juice against urinary tract infections became common practice and within the past 10-15 years, the body of scientific evidence to support that use has grown (*1*, *2*). Cranberry's status as a functional food has also grown as studies support its antioxidative properties (*3*) and potential to prevent cardiovascular diseases and certain cancers (*4*). Studies emerging in the past several years also show benefits for oral health (*5*). Recent studies in our laboratory suggest that cranberry's ability to inhibit microbes includes not only bacteria, but also human fungal pathogens. Cranberry's health benefits can be attributed to several classes of phytochemicals, many of which are also present in the foliage of the plant.

Phytochemical Constituents of Cranberry Fruit

Secondary metabolites in cranberry fruit are a diverse group that includes flavonoids, hydroxycinnamates and other phenolic compounds, organic acids, and isoprenoids. The flavonoids fall primarily into three classes: anthocyanins, flavonols and proanthocyanidins (tannins). Anthocyanin content in cranberry fruit has been reported as high as 91.5 mg per 100 grams of ripe fruit (*6*). The major anthocyanin glycosides are cyanidin-3-galactoside, cyanidin-3-arabinoside, peonidin-3-galactoside, and peonidin-3-arabinoside; cyanidin-3-glucoside and petunidin-3-galactoside have also been detected (*7*). Flavonol content ranges between 20-40 mg/100 g ripe fruit (*4*), with quercetin being the most abundant aglycone followed by myricetin and kaempferol. The major quercetin glycoside is the 3-O-galactoside (*8*, *9*). Quercetin arabinosides, xylosides and glucosides, as well as 6"-p-coumaroyl and benzoyl galactosides have been reported in cranberry extracts and powders (*8*, *9*). Polyflavan-3-ols, also known as proanthocyanidins (PACs) in cranberry fruit are primarily dimers, trimers and larger oligomers of epicatechin, containing two types of linkages between epicatechin units: the more common $4\beta \rightarrow 8$ (B-type) linkage (also found in apples, grape seed, and cacao), and a less common A-type linkage featuring both $4\beta \rightarrow 8$ and $2\beta \rightarrow O \rightarrow 7$ interflavanoid bonds that has been associated with the anti-bacterial adhesion properties of cranberry (*10*). Positions of the linkages may vary, so three-dimensional structures are diverse. 100 g of cranberry fruit typically contains 180 mg of oligomers having ten degrees of polymerization (DP) or less; the content of larger oligomers is estimated to be even higher (http://www.nal.usda.gov/fnic/foodcomp/Data/PA/PA.pdf.) (*11*).

Cranberry fruit also contains a wealth of phenolic and other organic acids, of which benzoic acid and p-coumaric (4-hydroxycinnamic) acid are the two major ones; others with additional aromatic hydroxylation are found (*12*). These phenolics, comprising about 0.6% of fresh weight, are present both in free form

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and esterified with sugars or other moieties. Trace amounts of resveratrol have been reported in cranberry juice, (*13*), but it does not make up a significant portion of cranberry phenolics. All of the phenolics can contribute to the antioxidant properties of the fruit. Anthocyanins in particular are known for their antioxidant power. Of the individual cranberry flavonoids tested, cyanidin-3-galactoside was the most effective in preventing lipoprotein oxidation (*8*).

Nonpolar constituents of cranberry fruit include isoprenoids and fatty acids. The major triterpenoid is ursolic acid, which exists both in its aglycone form and esterified with *cis* or *trans* p-hydroxycinnamic acid (*14*). LC-MS analysis estimated the total ursolic acid content at 65 – 120 mg per 100 g of fresh fruit depending on cultivar (*15*). The p-coumaroyl iridoid glycosides 10-*p-cis*- and 10-*p-trans*-coumaroyl-1S-dihydromonotropein were reported in cranberry juice (*16*), derivatives of iridoid glycosides previously reported in juice of *Vaccinium* berries (*17*). Cranberries also contain various carotenoids, the most plentiful of which is lutein (*18*). Cranberry seed oil contains various commonly occurring fatty acids, including myristic, palmitic, stearic, oleic, palmitoleic, linoleic, γ- linolenic, arachidic, lignoceric, behenic, gadoleic, erucic and nervonic acid, and some less common acids (pentadecanoic, 10-heptadecanoic, nonadecanoic acid, 11-trans-eicosenic, 11,14 eicosadienoic, 11, 14, 17 eicosatrienoic, and tricosanoic acids). Several omega-3 fatty acids including α-linolenic acid (ALA), eicosatrienoic acid, eicosapentaenoic acid (EPA), and docasapentaenoic acid (DPA) have also been reported (*19*). Various sterols (campesterol/brassicasterol, stigmasterol, β-sitosterol), phospholipids (phosphatidylinositol and phosphatidylcholine), as well as α-tocopherol and γ –tocopherol have also been reported in seed oil (*19*). Some representative structures of compounds identified in the fruit and leaves of *Vaccinium macrocarpon* are shown in Figure 1.

Constituents of Cranberry Foliage

Anecdotal evidence showing that insect pests are less likely to feed on certain cultivars of cranberry, and a lack of previous data on chemical constituents led us to investigate the phytochemical composition of the leaves. A feeding trial conducted with two common cranberry feeders, gypsy moth (*Lymantria dispar* L.) and flea beetle (*Systena frontalis* F.) on uprights of the Early Black and Howes variety showed the insects fed less often and less heavily on Early Black (*20*). Initial HPLC analysis of the constituents of foliage extracts indicated the presence of three classes of compounds: hydroxycinnamic acids, flavonol glycosides and tannins (likely epicatechin oligomers). The tannin profiles are complex and the subject of ongoing investigation. Procyanidin A2 was verified by comparison to commercial standard. The other water-soluble constituents were quantified as hydroxycinnamic acid or quercetin-3-galactoside equivalents. Comparison of cranberry foliage profiles from both cultivars harvested at a timepoint coinciding with gypsy moth infestation in 2006 showed significantly higher quantities of six compounds in the Early Black foliage (*20*). Two of these were quercetin glycosides, quercetin-3-O-galactoside and quercetin-3-O-rhamnoside (Table I), identified based on comparison to quercetin glycosides present in the fruit and commercial standards.

Isolation and identification of the other phenolic compounds was undertaken from an aqueous extract of Early Black foliage harvested in June 2008. Presence of the phenolic derivatives was confirmed by HPLC. The aqueous extract was fractionated by size exclusion chromatography on Sephadex LH-20 into five fractions. The first two fractions, eluting with 15:85 methanol/water and 50:50 methanol/water were composed of polar phenolic acid derivatives, and some later eluting phenolic acid derivatives respectively. Later fractions contained flavonol glycosides and proanthocyanidins based on HPLC-DAD analysis. From the 15:85 methanol/water fraction, four hydroxycinnamic acid derivatives (Table I) were isolated by preparative HPLC. Based on MS analysis and comparision to published data (*21*) identifying constituents in foliage of the blueberry *Vaccinium darrowi,* the four phenolic derivatives were identified (*20*). Two additional hydroxycinnamic acid derivatives were isolated from the 50:50 methanol/water fraction. These were tentatively identified as the coumaroyl iridoid glycosides 10-*p*-*trans*-coumaroyl-1*S*-dihydromonotropein and 10-*p*-*cis*-coumaroyl-1*S*-dihydromono-tropein, previously reported in cranberry fruit (*16*), based on ^{1}H NMR analysis and MS data.

Table I. Compounds identified in cranberry foliage

Structural class	*Significantly higher in EB*	*Compound identified*
Hydroxycinnamic acids	Yes	3-O-caffeoylquinic acid
	Yes	5-O-caffeoylquinic acid
	Yes	3-O-coumaroylquinic acid
	Yes	5-O-coumaroylquinic acid
Phenolic iridoid glycosides	ND	10-*p*-*trans*-coumaroyl-1*S*-dihydromonotropein
	ND	10-*p*-*cis*-coumaroyl-1*S*-dihydromonotropein
Catechins	ND	Procyanidin A2
Flavonol glycosides	Yes	Quercetin-3-O-galactoside
	No	Quercetin-3-O-xyloside
	No	Quercetin-3-O-arabinoside
	Yes	Quercetin-3-O-rhamnoside

ND = not determined

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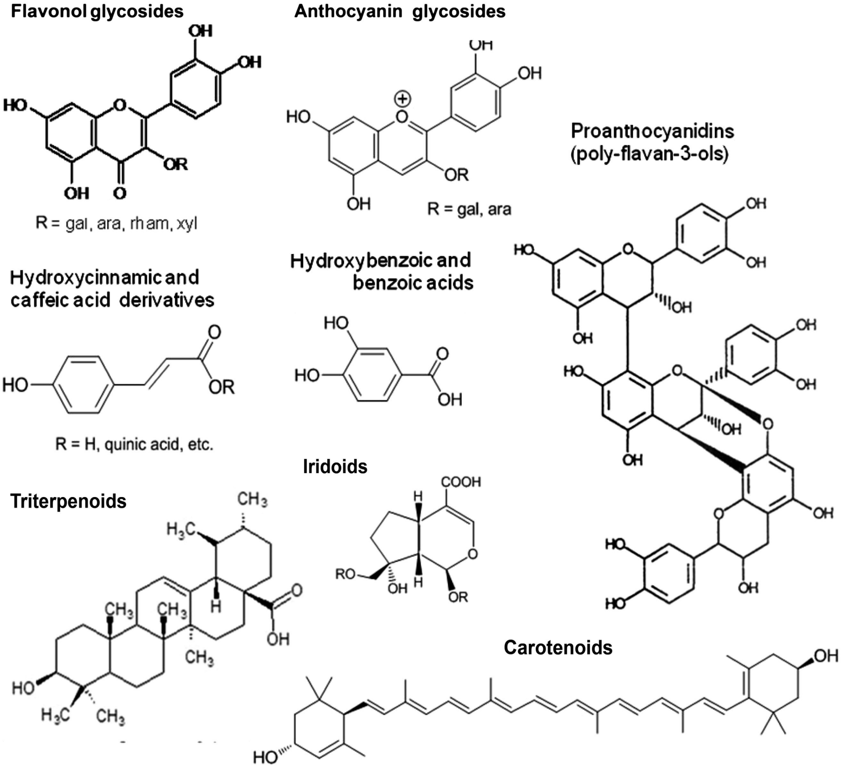
Flavonol glycosides
R = gal, ara, rham, xyl
Anthocyanin glycosides
R = gal, ara
Proanthocyanidins
(poly-flavan-3-ols)
Hydroxycinnamic and
caffeic acid derivatives
R = H, quinic acid, etc.
Hydroxybenzoic and
benzoic acids
Iridoids
Triterpenoids
Carotenoids

Figure 1. Some of the major phytochemical classes in fruit and foliage of Vaccinium macrocarpon.

Antioxidant and Anticancer Properties of Cranberry

Protection of the Cardiovascular System and Brain

Due to its high content of flavonoids and phenolic acids, cranberry ranks highly among fruit in both antioxidant quality and quantity (*3*), with the highest fresh weight polyphenol contents among over twenty fruits studied. These antioxidants are likely to play a role in combating conditions such as cardiovascular disease that are exacerbated by oxidative stress. Studies employing clinical, animal and *in vitro* models provide support for the role of cranberry in protecting against cardiovascular disease and associated conditions such as stroke, reviewed recently (*4*). Cranberry fruit, juice and constituents are thought to protect the vascular system by reducing lipoprotein oxidation, improving serum antioxidant status and blood lipid profiles, and mitigating the effects of oxidative stress and inflammation on vascular tissue and endothelial function. Clinical trials found improved lipid profile and serum antioxidant capacity in patients receiving cranberry juice (*22*, *23*). Patients on a two-week regimen showed reduced levels of oxidized LDL, inflammatory markers and adhesion molecules in circulation (*24*). Cranberry constituents also provided some measure of protection against necrosis or apoptosis to rat neurons under conditions of oxidative stress and glucose deprivation similar to those encountered during an ischemic stroke (*25*). Anthocyanins are likely to play a major role in the protective effects of fruit from the *Vaccinium* genus based on their combined antioxidant and antiinflammatory properties, and positive effect on vascular strength and flexibility.

Constituents with Anticancer Activities

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Several studies reporting the anti-cancer activities of cranberry polyphenolic extracts and individual cranberry constituents using in vitro models have appeared and have been reviewed recently (*26*). Collaborative studies between our research group and Canadian scientists have addressed the nature of compounds that inhibit tumor cell growth and proliferation and induce apoptosis in tumor cells, as well as the possible mechanisms of action of these compounds. Early studies by our group identified cis and trans-hydroxycinnamoyl esters of ursolic acid which inhibited the growth of several types of tumor cell including colon, breast, and prostate cell lines (*14*) These esters as well as the parent ursolic acid are plentiful in whole cranberry fruit (over 1.0 g total per kg fresh fruit weight) and occur to a lesser extent in other *Vaccinium* berries (*15*). Other compounds present in cranberry shown to inhibit tumor cell growth include quercetin and cranberry-derived proanthocyanidins. A proanthocyanidin fraction isolated from whole fruit that inhibited tumor cell growth was found by MALDI-TOF MS analysis to contain primarily oligomers of seven DP or less (*27*). Both ursolic acid and the whole cranberry fruit proanthocyanidins induced apoptosis in colon tumor cell lines (*28*). Studies using DU-145 prostate tumor cells have shown that whole cranberry polyphenolic extract induce apoptosis, accompanied by caspase-8 mediated cleavage of BID, increased PAR-4 protein, release of cytochrome C from the mitochondria, and activation of caspase-9 (*29*). In the same cell line, cranberry proanthocyanidins inhibited expression of matrix metalloproteinases -2 and -9, involved in the migration and metastasis of tumor cells. This inhibition occurred through multiple pathways including MAP kinase, PI-3 kinase and NF-kB (*30*).

In the past few years, animal model studies have begun to appear supporting the anticancer properties of cranberry. In a study of lymphoma, Balb/c female mice inoculated with lymphoma cells received injections of a high molecular weight cranberry NDM fraction at non-toxic doses every two days for two weeks. The treatment group showed no tumor development, whereas in the control group, 80% developed tumors in less than three weeks (*31*). An increase in production of antibodies against lymphoma cells was observed in treated mice. In a study of bladder cancer, Fischer-344 rats treated with nitrosamine to induce cancer received cranberry juice concentrate via gavage daily for up to six months. Treated mice showed a significant decrease in the number of bladder lesions, total weight of lesions, and number of carcinomas compared to control (*32*).

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Antimicrobial Activities of Cranberry

Prevention of Urinary Tract Infection Caused by *Escherischia coli*

The use of cranberry juice to prevent urinary tract infections has been supported through the years by anecdotal evidence, and a body of scientific evidence has emerged to validate this practice. The antibacterial effect was at first attributed to acidification of the urine, but studies correlating urinary pH with juice consumption did not support this claim (*1*). A series of investigations in the 1980's showed that cranberry juice could prevent adherence of *E. coli* bacteria to uroepithelial and other eukaryotic cells (*33*–*35*). In 1994, a clinical study of female residents of a long-term care facility found a significant decrease in bacteria in the urine after one month of cranberry juice consumption (*36*). Since then, at least fifteen clinical trials have evaluated the prophylactic effects of cranberry against urinary infections in a variety of populations; these have been reviewed (*1*, *2*) The efficacy of consuming cranberry juice or solids in UTI prevention for various populations has been demonstrated in women with recurrent UTIs, pregnant women, the elderly, and even children. Significant reductions in incidence and recurrence of UTI are observed in these populations when consuming cranberry.

The nature of the compounds conferring activity is the subject of active investigation. A high molecular weight, non-dialyzable material (NDM) from cranberry was first observed to inhibit adhesion of uropathogenic P-fimbriated *E. coli* or UPEC (*35*) but its composition is not well understood. Bioassay-guided fractionation of cranberry fruit targeting the active compounds found that cranberry PACs blocked adherence of UPEC to uroepithelial cells (*37*). Trimers of epicatechin with both A-type and B-type linkages were identified by NMR (*10*). Such A-linked PACs are common to *Vaccinium* fruit and this linkage may play a role in the activity. A comparison study of PACs isolated from several food sources found cranberry PACs far more effective at preventing *E. coli* adhesion than those from other sources (*38*). Anti-adhesion activity has also been detected in human urine following consumption of cranberry juice cocktail (*39*). The interactions between bacteria and cells in the presence of cranberry have been studied using atomic force microscopy to measure the effect of cranberry juice exposure on bacterial surface characteristics and adhesion forces in P-fimbriated *E. coli*. Exposure to cranberry juice brought about a decrease in fimbrial length and distribution of biopolymer density, as well as a decrease in adhesion forces in proportion to cranberry juice concentration (*40*). The effect on adhesion forces appears to be reversible (*41*). Other morphological changes and expression of associated genes have also been observed for *E. coli* bacteria grown in the presence of cranberry juice (*42*).

Oral Bacteria and Biofilm Prevention

The anti-bacterial adhesion properties of cranberry phytochemicals may also play a role in maintaining oral health. The pathogenesis of common oral infections, dental caries and periodontal diseases, involves an interaction of microorganisms with the tooth surface or the tooth-supporting tissues. Oral

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bacterial pathogens build up a structure known as a biofilm, allowing bacteria to firmly attach to oral surfaces. Studies have shown that cranberry extracts and fractions containing high-molecular weight constituents can inhibit growth, aggregation, and biofilm formation by oral pathogens including *Streptococcus mutans* and *Porphyromonas gingivalis,* and therefore limit infection by these organisms (*5*). The exact nature of the protective constituents from cranberry is still uncertain. The earliest studies used a high-molecular weight non-dialyzable material derived from cranberry juice by dialysis. This material was observed to inhibit coaggregation of numerous oral plaque-forming bacteria (*43*) as well as biofilm formation by *S. sobrinus* (*44*). The exact composition of this material has not been determined, but studies suggest that it contains proanthocyanidin oligomers that may aggregate in solution (*45*). A polyphenolic extract of cranberry juice likely to contain a variety of flavonoids inhibited biofilm formation in mixed cultures of *P. gingivalis* with *F. nucleatum* (*46*). Proanthocyanidin and flavonol fractions of whole fruit produced by chromatographic separation rather than dialysis inhibited biofilm formation by *S. mutans* (*47*). Thus, cranberry phytochemicals hold some promise for protection of oral health.

Protection against *Helicobacter pylori*

Helicobacter pylori infection is positively associated with the incidence of ulcers and gastric cancer (*48*); thus prevention of these infections may reduce gastrointestinal dysfunction and cancer risk. Anti-bacterial adhesion studies have demonstrated that in addition to inhibiting *E. coli* adhesion, cranberry components also inhibit adhesion of *H. pylori* to human gastric mucus (*49*) This finding was later supported by a randomized, double-blind placebo-controlled trial showing significantly lower levels of *H. pylori* infection in adults who consumed cranberry juice (*50*). In an *in vitro* study, cranberry polyphenolic extract had a bacteriostatic effect on the growth of *H. pylori* and induced morphological changes in bacteria (*51*).

Inhibition of Human Fungal Pathogens by Cranberry

While bacteria have been well-studied, comparatively little is known about whether cranberry can also protect against human fungal pathogens such as yeasts (*Candida* spp.) and others. We have investigated the effect of cranberry extracts (*Vaccinium macrocarpon*, var. Early Black) on the growth of several human fungal pathogens including *Cryptococcus neoformans*, *Candida krusei, Candida glabrata* and *Candida albicans.* These fungi have been associated with opportunistic human skin, oral and lung infections, especially in immunocompromised patients. Preliminary screening of whole fruit extracts using a 24-48 hour disk diffusion assay found that growth inhibition occurred in the presence of crude polyphenolic extract, phenolic acid and proanthocyanidin (PAC) fractions. A broth microdilution assay, NCCLS protocol M27-A3 (*52*), was employed to determine dose-response against the four pathogens. Growth of *C. krusei, C. glabrata* and *C. neoformans* over 48 hours was inhibited by whole fruit proanthocyanidin fractions at concentrations as low as 1 µg/ml (*53*).

Representative data is shown in Figure 2. *C. albicans* appeared to be much less susceptible to treatment than the other strains. MALDI-TOF MS analysis of the fractions indicated the presence of proanthocyanidin oligomers of up to 12 degrees of polymerization.

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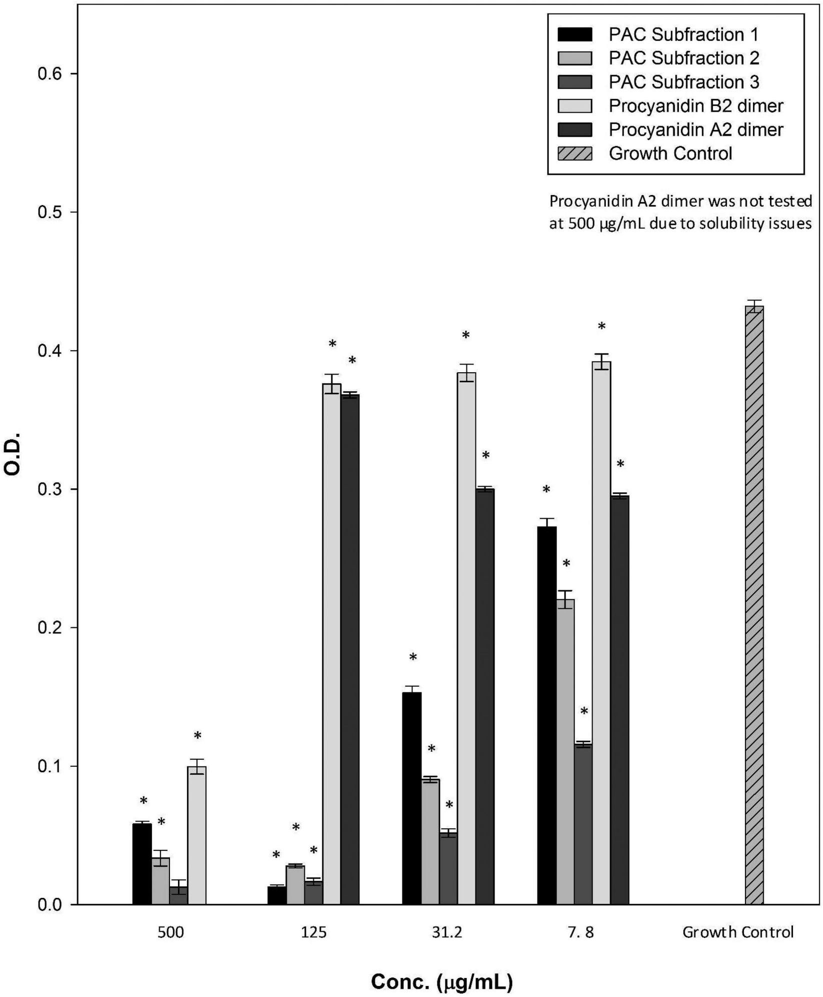

Figure 2. Effect of treatment with proanthocyanidin fractions from cranberry fruit (V. macrocarpon) or procyanidin dimers on the growth of Candida krusei over 48 hours. "" indicates treatment is significantly different from control, p < 0.001.*

The 50:50 methanol/water fraction isolated from cranberry foliage containing iridoid derivatives of p-coumaric acid was also evaluated against *C. glabrata* and *C. neoformans* using the broth microdilution assay. Growth of both species was inhibited at concentrations as low as 8 μg/mL. Representative data is shown in

Figure 3. These results suggest that various compounds in cranberry fruit and foliage, including proanthocyanidins and hydroxycinnamic acid derivatives, may possess anti-fungal properties and potentially offer protection against human pathogenic fungi. Structure-activity relationships and possible mechanisms of action are under investigation.

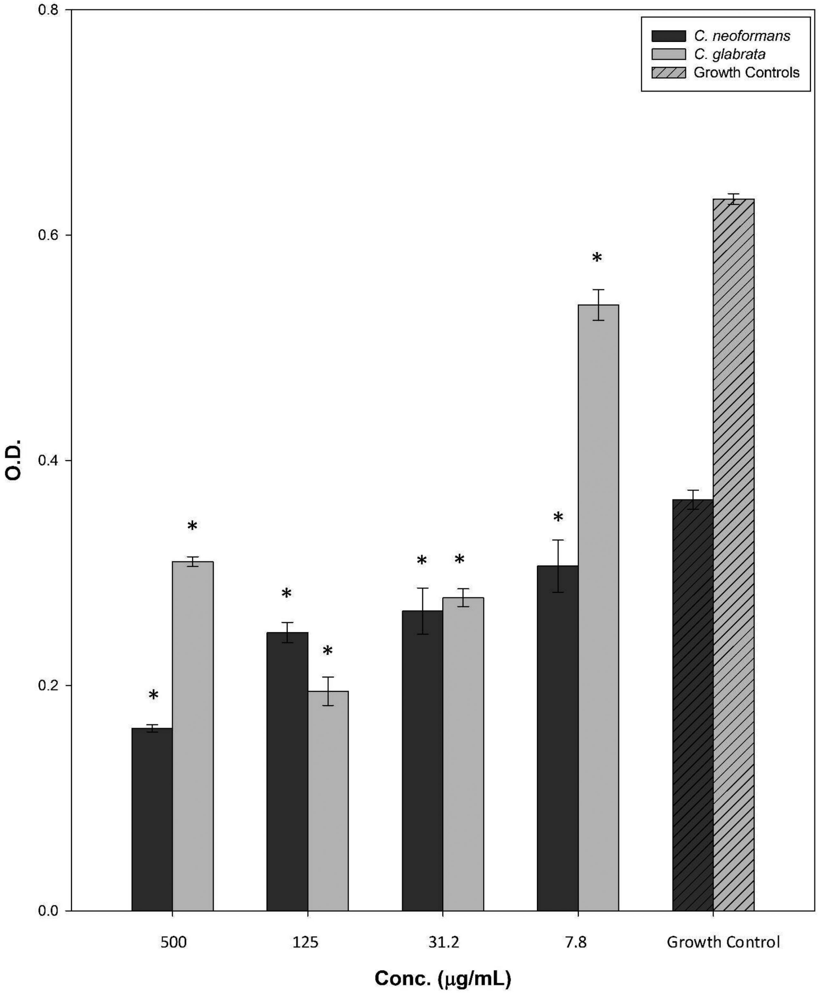

Figure 3. Effect of treatment with cranberry foliage phenolic extract on the growth of Cryptococcus neoformans (24 hours) and Candida glabrata (48 hours). "" indicates treatment is significantly different from control, $p < 0.001$.*

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Dual Protective Roles of Phytochemicals from *Vaccinium macrocarpon*

Phenolic compounds identified in the fruit and foliage of *V. macrocarpon* may serve a dual purpose in protecting the plant from herbivores as well as promoting health in the human diet . Cranberry fruit has been reported to contain 20-40 mg flavonols per 100 g fruit. Previous studies in our lab have found the total flavonol content in cranberry foliage (as quercetin) to be approximately 3%, varying slightly with cultivar, or 3 g per 100 g fresh leaf tissue (Nunnelley and Neto, unpublished results). Cranberry leaves are potentially an excellent source of many of the same flavonol glycosides found in cranberry and other fruits. Five quercetin monoglycosides known to be present in fruit were also detected in cranberry leaf extracts (Table I). Previous studies by our group and others have shown that cranberry flavonol glycosides are excellent antioxidants (*8*), decrease tumor cell proliferation, and mediate apoptosis in brain cells and prostate tumor cells (*25*, *29*) Quercetin has been found to modulate several pathways involved in cellular inflammatory response (*26*). Cranberry flavonols may also play a role in the inhibition of biofilm formation by *Streptococcus mutans* bacteria (*47*).

In addition to protecting the plant from ultraviolet light and other sources of environmental stress, cranberry flavonols and phenolic derivatives may play a role in pest resistance, particularly against gypsy moth. A study on pest deterrence in *Pinus* species suggested that feeding by gypsy moths (*Lymantria dispar* L.), a destructive pest of cranberry and other plants, is reduced in pine species due to the presence of flavonol glycosides. When quercetin and kaempferol glycosides isolated from *Pinus* spp. were incorporated into the diet of early instar gypsy moth larvae (*Lymantria dispar* L.) in controlled feeding trials, growth inhibition and increased mortality were observed (*54*). Iridoid glycosides were also shown to play a role in pest deterrence in a study of *Antirrhinum majus* L. where selectivity against gypsy moth was linked to the presence of two iridoid glucosides in the leaves (*55*).

Hydroxycinnamic acid is the major phenolic acid in cranberry fruit, and several derivatives have also been identified in the foliage. The content of these compounds as well as two quercetin glycosides was found to be significantly lower in foliage of Howes variety cranberry than in Early Black variety, and may therefore contribute to the observed decrease in feeding by certain insects (*20*). An extract of Early Black cranberry foliage containing hydroxycinnamic acid derivatives, which are tentatively identified as coumaroyl iridoid glycosides, inhibited the growth of the two human fungal pathogens *Candida glabrata* and *Cryptococcus neoformans,* as shown in Figure 3. Proanthocyanidin fractions from cranberry fruit also inhibited the growth of *Candida glabrata* and *Cryptococcus neoformans*, as shown in Figure 2. Such compounds are also likely to play a protective role in the plant. Ongoing studies in our lab suggest that cranberry-derived proanthocyanidins induce a stress response, the visible production of melanin, by several fungal plant pathogens that infect cranberry including *Phomopsis vaccinii, Colletotrichum acutatum*, *Fusicoccum putrefaciens* (*56*). Additional antimicrobial activities of these compounds and others derived from the cranberry plant are under investigation. We hope that such studies will lead to a better understanding of the relationships between phytochemical composition and both human and plant health.

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Chapter 6

Maqui Berry (*Aristotelia chilensis*) Juices Fermented with Yeasts: Effects on Phenolic Composition, Antioxidant Capacity, and iNOS and COX-2 Protein Expression

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Juices from the Chilean-grown maqui berry (*Aristotelia chilensis*) were fermented using two yeasts, *Saccharomyces cerevisiae bayanus* EC 118 (EC) and *Saccharomyces cerevisiae cerevisiae* RC 212 (RC), to investigate changes in phenolic phytochemical profile, antioxidant capacity, and anti-inflammatory potential. RC and EC fermentations both stabilized juice pH and reduced sugar content by 67% and 78% within 3 days, respectively. Initial concentrations of delphinidin 3-sambubioside-5-glucoside (41.0%), a major anthocyanin, were elevated up to 56.7% or 60.0% in EC or RC treatments, respectively, by day 9 of fermentation. Delphinidin 3, 5-diglucoside decreased from 24.5% to 9.5% (EC treatment) or to 8.6% (RC treatment) by day 9. Total polyphenol, proanthocyanidin, and hydrolysable tannins decreased over time in all treatments. Fermentation significantly increased

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gallic acid (a product of tannin hydrolysis) in EC treatment. Antioxidant capacity was enhanced with both yeasts, and EC fermented juice was able to inhibit iNOS and COX-2 protein expression at significantly lower concentrations compared to the unfermented juice. These results suggest that yeast fermentation changes polyphenolic composition in maqui juice in ways that could potentially improve health beneficial properties.

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Naturally-occurring polyphenolic phytochemicals from many edible plants have been linked to significant human health-protective benefits, and berries constitute a particularly rich dietary source of these bioactive compounds (*1*). The bioactivity of these small fruits has been frequently correlated with their phenolic content, and in particular, their anthocyanin constituents have demonstrated antioxidant, anti-inflammatory, and other therapeutic attributes.

Maqui berry (*Aristotelia chilensis* Stuntz), an edible black-colored fruit also known as wild Chilean black-berry, is popular for both food and medicine in Chile and western Argentina (*2*). The leaves and fruits of maqui berry have been used in folk medicine to treat a variety of ailments including sore throat, kidney pains, ulcers, fever, inflammation, and diarrhea (*3*). Research on phytochemical composition of the maqui berry has indicated the presence of phenolic acids, proanthocyanidins, and anthocyanins (*4*, *5*) . Maqui berry juice has shown antioxidant activity and capacity to inhibit low-density lipoprotein (LDL) oxidation in human endothelial cell cultures (*6*). The phenolic extract of maqui berry also showed aldose reductase inhibitory activity (*7*) and inhibition of lipid accumulation in an adipocyte cell line (*8*). The antioxidant activity of maqui extracts and subfractions was strongly correlated with polyphenol content, where the most active polyphenol-rich fractions were obtained using ethanol and acetone extractions (*4*, *8*). Maqui is one of the emerging novel exotic fruits from South America which has increasing appeal in the global marketplace (*9*), yet the effects of processing on bioactivity have not been characterized.

Chronic inflammation has been implicated in a wide range of age-related and other chronic diseases such as some types of cancers and cardiovascular diseases. Inducible forms of cyclooxygenase (COX-2) and inducible NO synthase (iNOS) are enzymes involved in the inflammation response. As an alternative to treatment with non-steroidal anti-inflammatory drugs, integrative research has recently focused on healthy lifestyle and dietary interventions for control of chronic inflammation. Berries constitute a particularly rich dietary source of these bioactive compounds (*1*, *7*), and several reports suggest that proanthocyanidins and anthocyanins in certain berry extracts can inhibit COX-2 activity (*8*, *10*).

The antioxidant activities of different phenolic compounds can be dependent on their structural characteristics (*11*). Data on polyphenol bioavailability is limited compared to drugs or to other components of the diet. For glycosylated polyphenols such as anthocyanins, the predicted effect of the attached moiety on passive diffusion across biological membranes suggests that the first step of metabolism would be the removal of sugar by certain enzymes (glycosidases). Glycosidase activity can occur in the food itself (endogenous or added during processing) or in the cells of the gastrointestinal mucosa or during secretion by the colon microflora (*12*). It is also well established that yeasts have enzymatic activities (β-glycosidase) that might hydrolyze glucosides. Therefore, fermentation with yeast as in wine-making might convert compounds to structurally-related products (*13*), which can be more bioavailable than the parent compounds (*14*).

To the best of our knowledge, phytochemical changes in maqui berry juice during fermentation have not been investigated nor have concurrent changes in antioxidant capacity and inhibition on iNOS and COX-2 protein expression been reported. We hypothesized that yeast fermentation of maqui berry juice would not only diminish sugar content, but also transform the polyphenolics of maqui berry juice into potentially more bioactive and bioavailable forms. The aims of the present study were: (1) to determine changes in phytochemical profiles (total polyphenols, anthocyanins, proanthocyanidins, hydrolysable tannins, and organic acids) for maqui berry juice after fermentation with two strains of wine yeast (*Saccharomyces cerevisiae*), and (2) to study the changes in antioxidant capacity and potential anti-inflammatory effects of fermented juice by monitoring protein expression of iNOS and COX-2 in a macrophage 264.7 cell line.

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Materials and Methods

Materials and Reagents

Maqui berries were collected in the Entrelagos region of Chile (S 40º 40’ 48, 5"/ W 72º 33’ 43, 3") during the ripening season of 2009, and shipped frozen by Fundación Chile (Santiago, Chile). Upon receipt, berries were immediately stored at -80 °C until use. Dry wine yeast Lalvin EC118 (*Saccharomyces cerevisiae bayanus*) and Lalvin Bourgorouge RC212 (*Saccharomyces cerevisiae cerevisiae*) were obtained from Lallemand Inc. (Montréal, Québec, Canada). All chemicals and reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Preparation and Fermentation of Maqui Berry Juice

Maqui berry juice was prepared as follows: after thawing for 30 min at room temperature, 200-g samples of maqui berries were blended with 200 mL water without breaking the seeds. Blended berries were filtered through Whatman No.1 filter paper (Whatman International Ltd, Maidstone, UK) under gentle vacuum. Yeasts EC118 (EC) and RC212 (RC) were rehydrated in 5 times their weight of potable water at 40 °C for 20 min with occasional gentle stirring to remove clumps prior to inoculation of the juice. Per manufacturer’s recommendations, RC or EC were inoculated at 40 or 50 mg dry yeast/100 mL, respectively, and held at 21 ± 1 °C during fermentation. Treatments of maqui berry juice included control (no inoculation with yeast), inoculation with RC yeast, or inoculation with EC yeast, with 4 replicates per treatment. Juice from all treatments was sampled on days 0, 1, 3, 5, 7, 9 and 11 of fermentation to monitor the changes in pH, sugar content, and phenolic composition. Samples were filtered through a 0.22 µm filter (Fisher Scientific, Hanover Park, IL) and frozen at -20 °C until further analysis.

pH and Phenolic Composition Analysis

The pH of the juice was measured using an Accumet AB15 pH meter (Fisher Scientific, Hanover Park, IL). Chemical composition of the juices was determined as follows. Total sugar content was determined using the phenol-sulfuric acid method using glucose as a calibration standard ($Y= 0.0093X + 0.012$, $R^2=0.99$). Total phenolic content was quantified by the Folin-Ciocalteu method using gallic acid as a calibration standard ($Y= 0.019X + 0.033$, $R^2= 0.99$). Both proanthocyanidins (PAC) and hydrolysable tannins were determined with the vanillin method using methanol and glacial acetic acid, respectively, following the methods of Waterman and Mole (*15*) with (+) - catechin (Sigma- Aldrich, St. Louis, MO) as a calibration standard. The equations for PAC and hydrolysable tannins were $Y = 0.0128X - 0.0073$ ($R^2 = 0.97$) and $Y= 0.1028X - 0.0092$ ($R^2 = 0.99$), respectively. All prepared samples were read with a spectrophotometer (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA). The wavelengths for assay of total sugar, total phenolics, PAC and hydrolysable tannins were 490, 760, 500 and 500 nm, respectively.

Quantification of Anthocyanins (ANC)

Analysis of ANC content in filtered juice was conducted on an 1100 HPLC (Agilent Technologies, Santa Clara, CA) using a reversed-phase Supelcosil-LC-18 column (250 mm × 4.6 mm × 5 µm) (Supelco, Bellefonte, PA). The mobile phase consisted of 5% formic acid in H_2O (A) and 100% methanol (B). The flow rate was 1 mL/min with a step-wise gradient of 10%, 15%, 20%, 25%, 30%, 60%, 10%, and 10% of solvent B at 0, 5, 15, 20, 25, 45, 47, and 60 min, respectively. All samples for HPLC were filtered through a 0.22 µm syringe filter unit prior to injection. Chemstation software (Agilent Technologies) was used for both protocol control and data processing. Quantification of ANC was made using cyanidin-3-*O*-glucoside (Polyphenols Laboratories AS, Sandnes, Norway) as an external standard at six concentrations of 0.025, 0.05, 0.1, 0.25, 0.5 and 1.0 mg/mL. ANC concentrations were expressed as cyanidin-3-*O*-glucoside equivalents.

Quantification of Organic Acids by HPLC

The organic acid content was determined following the methods of Singh and Graeber (*16*) with slight modifications. Filtered juice was injected into an ion exclusion column (Aminex HPX-87H, Bio-Rad, Hercules, CA) maintained at 35 °C. Organic acids were eluted from the column with HPLC grade water containing 5 mmol/L sulfuric acid. The elution rate was 0.6 mL/min with UV detection at 210 nm.

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Determination of Antioxidant Capacity

Antioxidant capacity was measured by the oxygen radical absorbance capacity assay (ORAC) as recently described for maqui extracts (*8*) Briefly, aliquots of 20 µL sample, Trolox standard dissolved in 75 mmol/L phosphate buffer pH 7.4 or 75 mmol/L phosphate buffer pH 7.4 blank were added to a 96-well black walled plate. This was followed by the addition of 120 µL of 17 nmol/L fluorescein. The plate was then incubated for 15 min at 37 °C followed by the addition of 60 µL of 153 mmol/L AAPH. The plate was read in a fluorescence plate reader, FLx800tbi (Bio-Tek, Winooski, VT), at 37 °C, and read every 2 min for 120 min with excitation 485 and emission 582 nm. Results were expressed as µmol/L Trolox equivalents.

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Inhibition of COX-2 and iNOS Protein Expression

All treatments were assayed for cytotoxicity before any treatment was performed. CellTiter 96AQueous One Solution was used to determine the number of viable cells according to the manufacturer's manual (Promega, Madison, WI). Briefly, the CellTiter 96AQueousOne Solution (20 µL) was added to 100 µL of media or sample contained in wells (with cells), and then the plate was incubated in a 5% CO_2 incubator at 37 °C. After 2 h, absorbance was measured at 515 nm with a 96-well plate reader (Biotek Instruments).

Macrophage cell line RAW 264.7 was seeded at 2×10^5 cells/well in 6-well plates and cultured in FBS/DMEM medium at 37 °C in a humidified atmosphere with 5% CO_2/95% air. Cells that yielded more than 85% viability of the cytotoxicity test were used for the treatments. After 24 h of incubation, cells were treated with juices at the final concentrations of 12.5, 25, 50, 100, 200 and 400 µmol/L gallic acid equivalents (GAE) as determined by the Folin Ciocalteu assay and 1 µg/mL of LPS. Cell lysates were used to study the effect of the fermented maqui berry juices on the protein expression of COX-2 and iNOS.

Protein expression of COX-2 and iNOS was determined in cell lysates by the Western Blotting method. Briefly, treated cells were washed with ice cold DMEM and ice cold phosphate buffered saline before treatment with 200 µL of Laemmli buffer (Biorad Laboratories, Hercules, CA) and with 5% β-mercaptoethanol lysing buffer. After lysis, the cell lysates were boiled for 5 min and approximately 25 µg of proteins were loaded on 4–20% Tris-HCl ready gels (Biorad Laboratories) for protein separation. Separated proteins were transferred to PVDF membrane and blocked with 5% nonfat dry milk in 0.1% Tris-buffered saline Tween 20 (TBST) for 1 h at 4 °C. After blocking, the membrane was washed with 0.1% TBST (5 times, 5 min each) and incubated with either COX-2 or iNOS mouse monoclonal antibody (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 °C overnight. The membrane was washed again and incubated with anti-mouse IgG horseradish peroxidase conjugate secondary antibody (GE Healthcare, Amersham, UK) for 3–4 h at room temperature. After incubation and repeated washing, the protein expression of COX-2 and iNOS was visualized using chemiluminescent reagent (GE Healthcare) following manufacturer's instructions. The membrane was imaged with a Kodak Image

Station 440 CF. The expression of these enzymes was calculated as the ratio between COX-2 or iNOS and actin band intensity (%).

Statistical Analysis

Data were analyzed using ANOVA (SAS 2009, Version 9.2). Treatments were compared based on least significant differences (LSD) using statistical SAS analysis software. Significant differences were reported at P values < 0.05 and <0.01.

Results and Discussion

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Changes in pH for all maqui berry juice treatments were similar during the first 3 days of treatment, decreasing from 4.0 to 3.6 (Figure 1). At day 5, both yeast treatments (RC and EC) exhibited a slight increase in pH to 3.8 while the control treatment pH continued to decline. After day 5, the pH of yeast treatments declined but at a slower rate than the control. Ultimately over the entire period, yeast treatments maintained a more stable pH only declining from 4.0 to 3.6 ($P > 0.05$) while the controls fell to pH level 2.9. During the entire fermentation period, no significant differences in pH were noted between the two yeast treatments (Figure 1). It has been reported that *S. cerevisiae* tends to be a dominant species during fermentation at the pH range from 3.8 to 4.2, as it is able to inhibit wild yeasts (*17*). The dramatic fall in pH for the control treatment during the test period was likely caused by the natural microflora present on the surface of the fruits, since the juices were not sterilized.

Structural transformations of anthocyanins are influenced by pH, therefore a more stable pH should help preserve anthocyanin structure. Anthocyanins in elderberry were found to be stable at pH 3.5 and the phenolic content of cranberry juice fermented with bacteria remained very stable at pH 4.0 (*18*)

Changes in Total Sugar Content

The initial total sugar content of maqui juice averaged 39.18 g/L, but significantly decreased by the third day in RC and EC treatments by 66.9% and 78.2%, respectively (Figure 2), while the control (not inoculated) showed no significant decline in sugar content. No further decline in sugar content occurred between days 3 and 11 in both yeast treatments. Fermentation of the sugars and other nutrients by endogenous yeast leads to alcohol and organic acid production (*19*). However, total initial sugar content of maqui berry juice (39.18 g/L) was fairly low compare with that of grape musts, which have fermentable sugar ranges between 125 and 250 g/L (*20*), Theoretically, 1g of sugar can be converted into 0.5 g alcohol during yeast fermentation, and therefore the final alcohol concentration of maqui juice was less than 2% in fermented samples.

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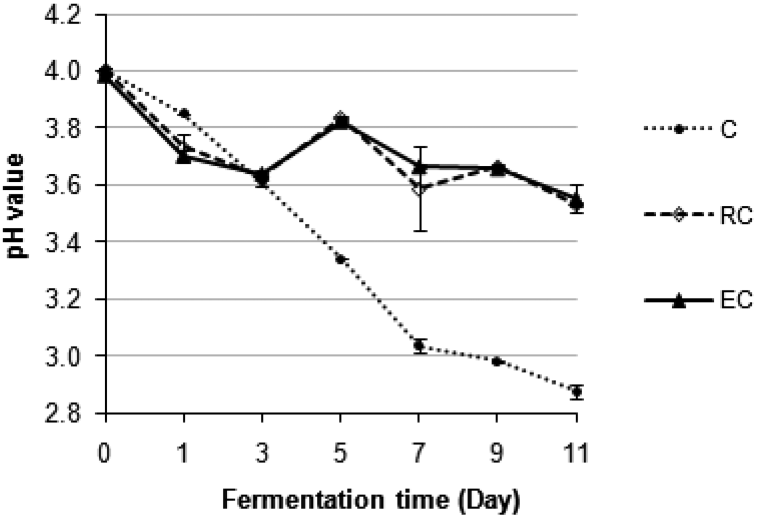

Figure 1. pH changes of maqui berry juice during fermentation. C = Maqui berry juice without inoculation (Control); RC = Maqui berry juice inoculated with yeast RC; EC = Maqui berry juice inoculated with yeast EC. Data are expressed as means ± SD.

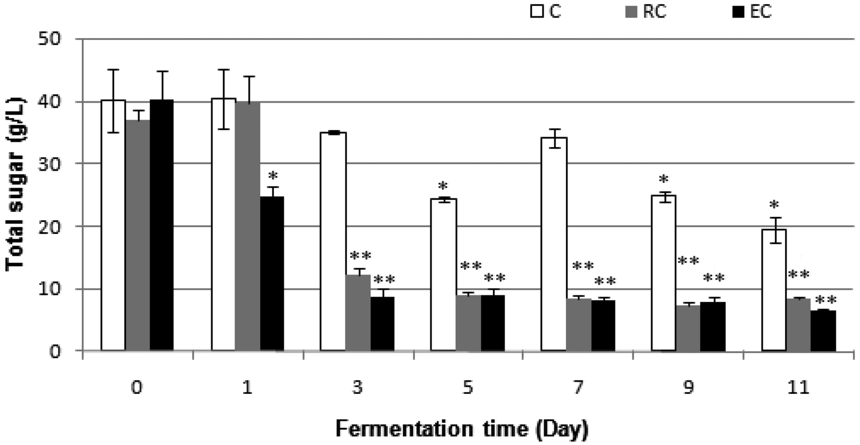

*Figure 2. Total sugar content changes in maqui berry juice during fermentation. C = Maqui berry juice without inoculation (Control); RC = Maqui berry juice inoculated with yeast RC; EC = Maqui berry juice inoculated with yeast EC. Sugar content was measured as glucose equivalents. Data are expressed as means ± SD. *P < 0.05, ** P<0.01 compared to day 0.*

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Previous investigations on therapeutic and anti-inflammatory properties of berry compounds have relied on organic solvents (e.g. ethanol, methanol and acetone) to extract and prepare the most active polyphenol-rich fractions (*4*). Sugars are usually removed from the extract using solid-phase extraction (SPE) with resin and methanol solvent (*21*, *22*) to avoid dilution of the active compounds, and/or interference by sugars in bioassays (*23*). However, the removal of sugars using SPE also can reduce the final polyphenolic content by 20% (*21*). In addition, the SPE methods include the expense of both the resin and the environmentally-destructive organic solvents, and the potential for harmful solvent residues to remain in the final products.

The removal of sugars is a necessary step to prevent sugar interference during bioactivity assessment (*21*), since high levels of sugars may result in lower antioxidant activity Some naturally-occurring plant phenolics exist in conjugated glycosylated forms which can interfere with their ability to serve as antioxidants (*22*). Lowered antioxidant capacity can translate into reduced health functionality when these phenolics are ingested by humans (*1*, *24*). Hydrolysis of these phenolic glycosides appears to be an attractive means of releasing free phenolic compounds thereby improving the antioxidant capacity and health functionality of phytochemicals (*25*). Compared to the traditional way of removing sugar by SPE (*21*), controlled fermentation could provide a commercially applicable method to economically remove sugars and potentially improve the antioxidant capacity of maqui berry juice within a relatively short period.

Changes in Phenolic Composition of Maqui Berry Juice During Fermentation Anthocyanin Content

The moisture content of the juice prepared in our study was about 87%, and approximately 1L juice was obtained from 1 kg of maqui fruit. Even after dilution of the juice by 50% during this juicing protocol, the anthocyanin concentration of maqui juice averaged 4,558 mg/L. This vastly exceeded reported values for blueberry juice (9.7 - 99.6 mg/L) (*26*), cranberry juice (77 mg/L) (*27*) juice (580 mg/L) (*28*) and strawberry juice (110 mg/L to 270 mg/L) (*29*). After a single water extraction, maqui yielded 960 mg anthocyanin (C3G equivalents)/100g dry fruit, which exceeds reported values for wild blueberry (558.3 mg/100g), raspberry (365.2 mg/100g), chokeberry (177.4 mg/100g) and strawberry (97.5 mg/100g) (*30*) even though a thorough, repeated extraction with acidified methanol was performed to obtain anthocyanins in each of these cited examples.

Anthocyanin concentrations were elevated in the control, RC and EC treatments by 41.3, 48.6 and 50.8%, respectively, after one day of fermentation (Figure 3A). By day 3, the anthocyanin content of RC and EC treatments remained significantly higher than the initial values (20.1% and 35.1%, respectively), while the anthocyanin content in the control was not significantly different (Figure 3A). These results agree with other reports where anthocyanin pigment content increased in a fermentation broth during the initial days of treatment (*31*, *32*). For the control, slight fermentation by natural microbes on the surfaces of the fruits may be responsible for initial elevation in pigments, whereas in the yeast inoculated treatments, *S. cerevisiae* was the dominant microbial species.

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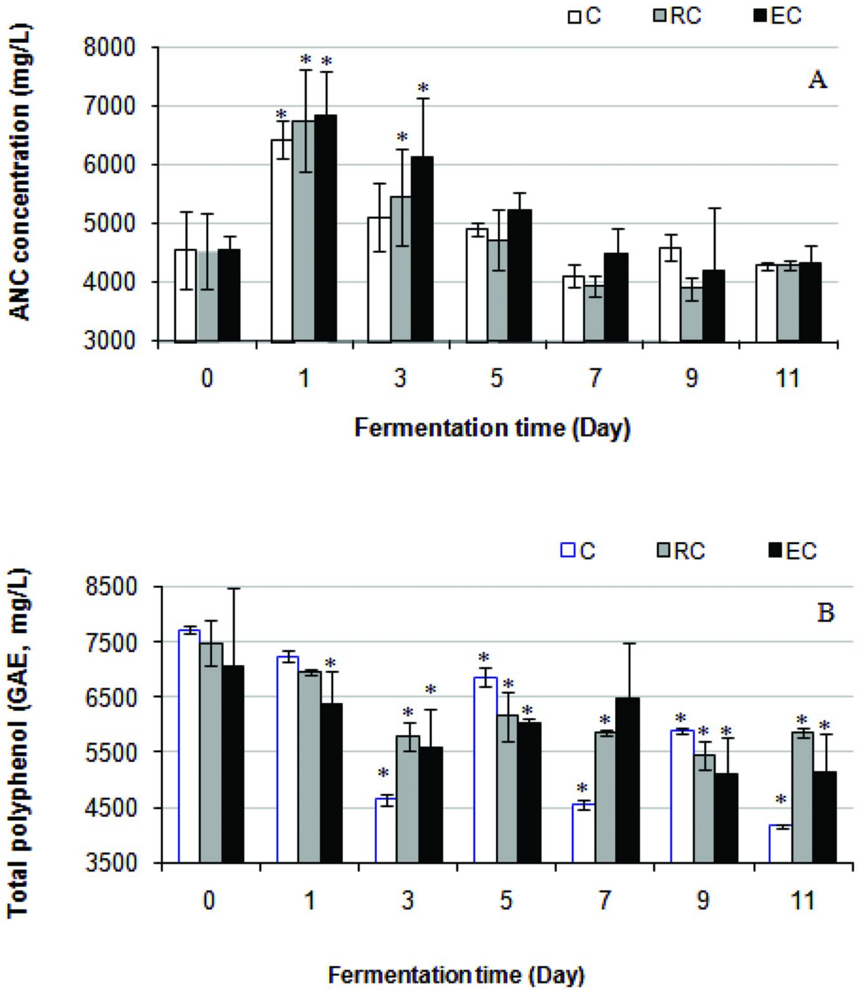

*Figure 3. Total polyphenol and anthocyanin content changes in maqui berry juice during fermentation. C = Maqui berry juice without inoculation (Control); RC = Maqui berry juice inoculated with yeast RC; EC = Maqui berry juice inoculated with yeast EC. Anthocyanin (ANC) content was described as cyanidin-3-O-glucose equivalents and total polyphenol content was measured as gallic acid equivalents (GAE). Data are expressed as means ± SD. *P < 0.05 compared to day 0.*

Anthocyanins can undergo significant degradation when fruit is processed (*33*) . The loss of anthocyanin in typical juice processing may be considerable; for example, only 50% of the anthocyanins from strawberry and blueberry may be recovered during juice pressing, and during storage of the juice (*34*), the anthocyanin content may decrease even further. During the storage and ripening of wines, considerable losses of anthocyanins can also occur, for example a young red wine with approximately 500 mg anthocyanin/L may be reduced to 200 mg/L at maturity (*35*). To optimize color and market appeal, it is desirable to find ways to prevent degradation of anthocyanins during processing and storage.

Total Polyphenol Content

Total polyphenol (TP) content in the maqui berry juice prior to fermentation averaged 7,413 mg gallic acid equivalent/L (Figure 3 B). During fermentation, the TP for all three treatments decreased significantly by day 3. While TP content of the control continued to fluctuate, the TP content in the yeast-inoculated treatments exhibited no significant changes during the remainder of the fermentation. No significant differences occurred between the groups inoculated with either yeast treatments, RC and EC, at any sampling time.

Similar to anthocyanins, total phenolic (TP) content may also undergo significant degradation when fruit is processed (*33*). Multiple chemical modifications in anthocyanin and overall phenolic profiles have been reported during the process of wine fermentation (*36*) Su & Chien (*26*) indicated that TP was not affected by 3-5 days yeast fermentation of blueberry juice, but in most yeast fermentation processes, TP tends to be lower by the end of a fermentation run (*31*) as in our observed results. In contrast, bacterial fermentation of a wild blueberry juice and broth (1:1 v/v ratio) mixture resulted in a significant increase in TP content (*37*). However, in these reports, the methodology was not traditional fermentation, in that liquid nutrient medium was incorporated into the juices during the process.

In our study, by day 3, the TP in the control, RC, and EC treatments decreased by 39.7, 22.6, and 20.7%, respectively. Over these same initial days of the fermentation cycle, the anthocyanin content increased, but by day 3, the increase was only significant for the RC and EC yeast treatments (20.1 and 35.1%, respectively). Simultaneously, the sugar content in the control, RC, and EC treatments decreased by 12.7, 66.9 and 78.2%, respectively. These changes in anthocyanin and TP are consistent with reported trends, which also indicated that during grape fermentation, anthocyanin levels may be elevated between days 1 and 3 of fermentation (*32*), and TP levels after fermentation were lower than initial values in juice (*31*). Anthocyanins contribute substantially to antioxidant potential (*26*, *38*) but high levels of sugars result in diminished antioxidant activity (*22*), therefore the stabilizing influence of fermentation on anthocyanin content and TP, while lowering sugar concentrations, has the potential to improve the antioxidant capacity of the fermented maqui juice product.

Proanthocyanidin and Hydrolysable Tannin Content

The proanthocyanidin (PAC) content of the three treatments (control, RC, and EC) was much higher (about 6-fold) than hydrolysable tannin content (Table 1). The PAC and hydrolysable tannins were lower on day 3 compared to their initial content. After day 3, the PAC and hydrolysable tannin remained relatively stable in the two groups inoculated with yeast. Previous studies showed that there was no significant correlation between PAC content and antioxidant values in wines (*13*),

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therefore, the decrease of PAC was not expected to adversely affect the antioxidant properties of maqui berry juices.

The pathways involved in tannin degradation by yeast have not been well defined, but it is possible that their mechanism(s) of degradation may be similar to that of fungi, in which gallic acid degradation is the main pathway (*39*). Since PAC constituted a significant proportion of the phenolic content (*40*), the conversion of the interior monomeric unit (of PAC) to the corresponding ANC during acid-catalyzed depolymerization (*41*) may be one of the factors causing the anthocyanin levels to be elevated during initial days of fermentation in our study. Additionally, the decline in PAC content over time likely contributed to the decrease in TP observed during fermentation.

Organic Acid Profiles

The main organic acids identified in maqui berry juices included quinic, citric, malic, chlorogenic, succinic and gallic acid. This was verified using LC-MS and HPLC spectra, commercial standards, and by comparison with published data (Figure 4A). No differences between treatments were noted in the main types of organic acids detected in maqui berry during fermentation (Figure 4A, B). Compared to day 0, the gallic acid increased by 86.6% on day 1 and 110.6% on day 3 in the EC treatment ($P < 0.01$) (Figure 4C). Gallic acid is the product of tannin hydrolysis (*42*). The production of gallic acid observed during blueberry fermentation by bacteria could be caused by hydrolysable tannin degradation (*37*). Gallic acid plays an important role in antioxidant capacity of berries and shows a dose-dependent inhibition effect on the production of NO (*43*). Therefore, the decrease of hydrolysable tannins and increase of gallic acids of maqui berry juice in our study could be attributed to yeast degradation of hydrolysable tannins through the gallic acid degradation pathway.

Anthocyanin Profile

Maqui berry extract contained seven major anthocyanin compounds including delphinidin-3-sambubioside-5-glucoside, delphinidin-3, 5-diglucoside, cyanidin-3-sambubioside-5-glucoside, delphinidin-3-sambubioside, delphinidin-3-glucoside, cyanidin-3-sambubioside, and cyanidin-3-glucoside. This was in agreement with previous reports (*4*, *5*, *8*) . Delphinidin 3-sambubioside-5-glucoside was the dominant component of maqui berry juice, and accounted for 41.0% of the total anthocyanins, followed by delphinidin 3, 5-diglucoside with 24.5% (Table 2, Figure 5). Previously, after extraction with methanol, delphinidin 3-sambubioside-5-glucoside and delphinidin 3, 5-diglucoside were the two primary components, accounting for 33.7% and 17.2% of the total anthocyanin, respectively (*5*). However, when maqui was extracted with ethanol, delphinidin-3-glucoside was the main anthocyanin component (30%), while delphinidin 3-sambubioside-5-glucoide and delphinidin 3, 5-diglucoside accounted for 14.3% and 12.8%, respectively (*8*). Cyanidin 3-sambubioside had the least concentration in maqui berry juice, 0.23% (Table 2, Figure 5), with a comparable level in the ethanol extract (0.5%) from our previous study (*8*).

Various combinations of ethanol, methanol and acetone have been used in fruit sample preparation, while methanol and acetone are much more efficient for flavonoid extractions than ethanol (*44*). Typically, water alone is less efficient than water mixed with organic solvents to extract the anthocyanins. In this study, water extraction (juice) during fermentation effectively extracted both delphinidin 3-sambubioside-5-glucoide and delphinidin 3, 5-diglucoside.

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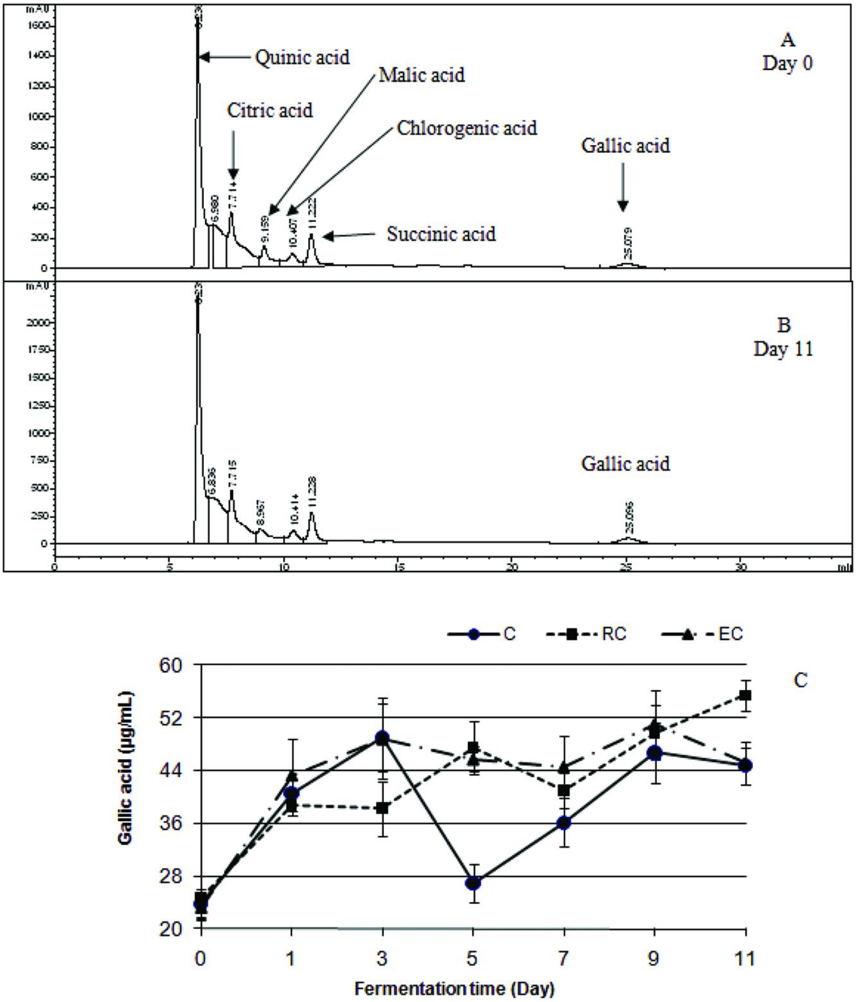

Figure 4. HPLC profiles of organic acids and gallic acid content of maqui berry juice during the fermentation. C = Maqui berry juice without inoculation (Control); RC = Maqui berry juice inoculated with yeast RC; EC = Maqui berry juice inoculated with yeast EC. Data are expressed as means ± SD.

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Table 1. Changes in proanthocyanidins (PAC) and hydrolysable tannins during the fermentation of maqui berry juice

		Fermentation Time (Day)						
		0	*1*	*3*	*5*	*7*	*9*	*11*
PAC (µg/mL)	C	2409.1 ± 118.2	2123.9 ± 278.4*	1620.8 ± 243.1*	1624.7 ± 317.1*	1606.7 ± 201.1*	2059.1 ± 138.1*	1363.0 ± 79.6*
	RC	2124.7 ± 274.3	2102.0 ± 194.3	1502.4 ± 116.2*	1688.4 ± 194.3*	1744.6 ± 134.9*	1595.4 ± 162.1*	1496.6 ± 111.6*
	EC	2597.7 ± 122.7	2061.8 ± 103.3*	1698.1 ± 383.7*	1709.8 ± 67.0*	1657.9 ± 19.3*	1764.1 ± 185.3*	1339.1 ± 88.8*
Hydrolysable tannins (µg/mL)	C	371.6 ± 2.7	350.6 ± 4.7	239.1 ± 63.4*	285.3 ± 5.2*	262.8 ± 0.5*	342.0 ± 7.5	232.0 ± 2.7*
	RC	343.3 ± 18.0	322.2 ± 14.9	276.2 ± 13.9*	283.1 ± 12.0*	277.6 ± 8.4*	253.1 ± 7.9*	260.6 ± 11.4*
	EC	407.9 ± 29.3	322.6 ± 46.1*	267.6 ± 25.0*	270.1 ± 0.5*	266.0 ± 6.5*	264.2 ± 3.6*	204.1 ± 7.5*

Note: C=Maqui berry juice without inoculation (Control); RC= Maqui berry juice inoculated with yeast RC; EC= Maqui berry juice inoculated with yeast EC; The PAC and hydrolysable tannins content were measured as (+) - catechin equivalents. Data are expressed as mean ± SD. *$P < 0.05$ compared to day 0.

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Table 2. Percentages of anthocyanins (%) in maqui berry juice during fermentation by yeast EC118 and RC 212. Day 0 = (fresh squeezed) unfermented juice, RC3 and RC9 – inoculated with RC yeast and sampled on the third day or ninth day, respectively; EC3 and EC9 - inoculated with EC yeast and sampled on the third day or ninth day, respectively

		(% of individual anthocyanin)				
Peak	*Anthocyanin*	*Day0*	*RC3*	*RC9*	*EC3*	*EC 9*
1	Delphinidin 3-sambubioside-5-glucoside	41.0	54.6	59.6	51.8	56.7
2	Delphinidin 3,5-diglucoside	24.5	12.0	8.6	12.4	9.5
3	Cyanidin 3-sambubioside-5-glucoside	17.0	19.1	19.9	19.5	20.8
4	Delphinidin 3-sambubioside	5.2	7.0	6.7	7.4	6.8
5	Delphinidin 3-glucoside	8.2	4.5	2.8	5.6	3.5
6	Cyanidin 3-sambubioside	0.23	0.15	0.14	0.29	0.24
7	Cyanidin 3-glucoside	3.9	2.6	2.2	3.0	2.5

The anthocyanidins found in higher plants are cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin, with a distribution in nature of 50%, 12%, 12%, 12%, 7% and 7%, respectively (*45*). In maqui berry juice, delphinidin accounted for 78.9% of the anthocyanidin, and cyanidin comprised the remainder. Sambubioside and glucoside comprise the sugar components of anthocyanidins from maqui berry. Glucose, galactose and arabinose, which are usually conjugated to the anthocyanidin molecule via the C3 hydroxyl group in ring C, are the most common sugars for anthocyanins in the glycosidic forms (*46*). During fermentation, the percentage of the seven compounds intrinsic to maqui berry changed dramatically (Table 2, Figure 5). Delphinidin 3-sambubioside-5-glucoside levels increased from 41.0% on day 0 to 60.0% (RC9) and 56.7% (EC9) on day 9. Meanwhile, the content of delphinidin 3, 5-diglucoside decreased from 24.5% to 8.6% (RC) and 9.5% (EC) by day 9, and delphinidin 3-glucoside decreased from an initial 8.2% to 2.8% (RC) and 3.5% (EC) by day 9. No changes were observed in the number and type of anthocyanins in maqui berry juice through the fermentation process, however, the change in the individual anthocyanin proportions in the extracts during fermentation resulted in a different anthocyanin concentrations compared to the unfermented juice. The yeasts have enzymatic activities (β-glucosidase) that might produce the hydrolysis of anthocyanins into anthocyanidins and free the glucoside molecules (*25*).

Numerous studies have suggested a correlation between berry health benefits and phenolic composition (*12*). However, the bioactivity of berries can vary depending on the profile and concentration of the phenolic compounds present. Elevation of individual anthocyanin concentrations as well as proportional changes during fermentation of maqui berry juice might result in new health benefits of maqui berry. Cespedes et al. (*2*, *4*) found that ethanol, methanol, and acetone extracts of maqui berry showed radical scavenging ability and lipid peroxidation inhibition in vitro. Because bioactive potential of fermented maqui berry juice or products have not been reported, we examined these treatments in terms of ORAC activity and anti-inflammatory capacity.

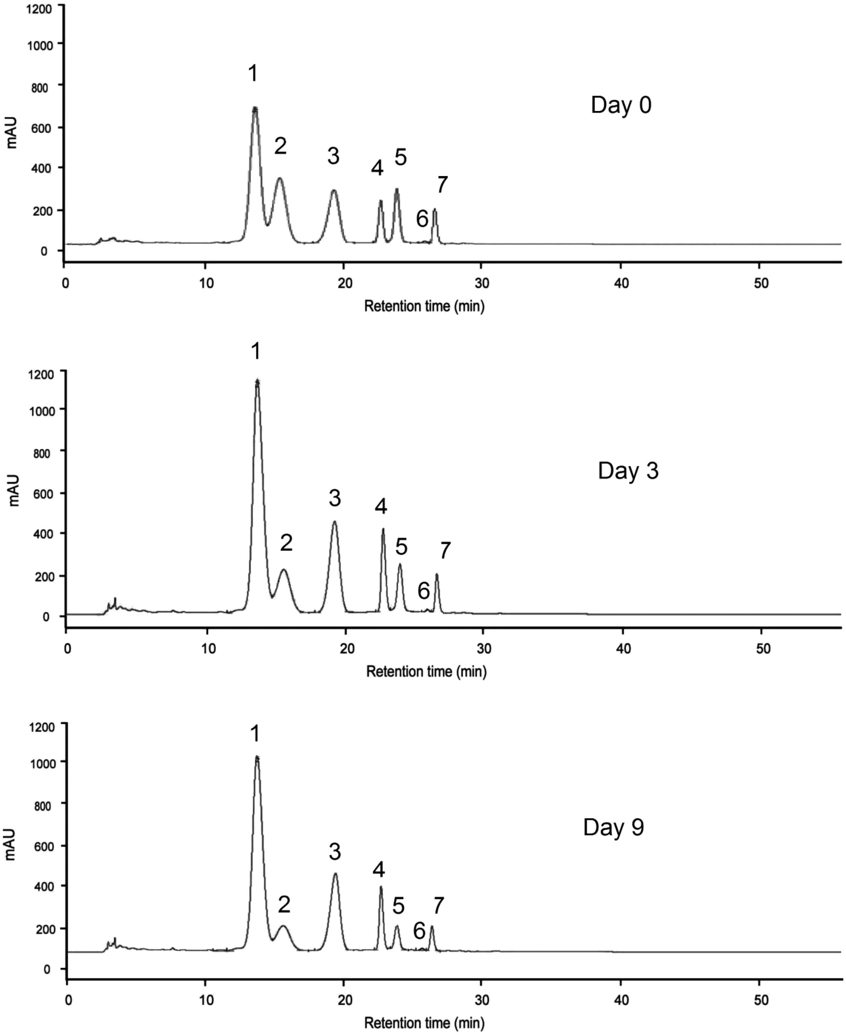

Figure 5. Representative HPLC chromatogram of anthocyanins in maqui berry juice during fermentation by yeast EC118 at day 0, day 3 and day 9. Peak identification: (1) delphinidin 3-sambubioside-5-glucoide, (2) delphinidin 3, 5-diglucoside, (3) cyanidin 3-sambubioside-5-glucoside, (4) delphinidin 3-sambubioside, (5) delphinidin 3-glucoside, (6) cyanidin 3-sambubioside, (7) cyanidin 3-glucoside.

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Antioxidant Capacity

The initial ORAC value of juices at 50 µmol/L and 100 µmol/L GAE were 59.2 (data not shown) and 125.2 µmol/L Trolox equivalent, respectively (Figure 6). ORAC values in maqui juice doubled, as the TP content doubled, indicating a strong correlation between antioxidant capacity and TP content. These results were in agreement with previous reports (*13*), which indicated a strong correlation between ORAC values and TP content in red wine. ORAC values for fermented maqui berry juice (EC treatment) increased dramatically compared to the corresponding unfermented juice at both concentrations ($P < 0.01$). Juice inoculated with EC yeast had a higher ORAC value than juice inoculated with RC yeast, which indicated that EC may have more potential to increase the bioactivity of maqui berry juice. Therefore, juice fermented by EC was selected to assess the potential anti-inflammatory capacity, via inhibition of COX-2 and iNOS protein expression in a macrophage cell line, below.

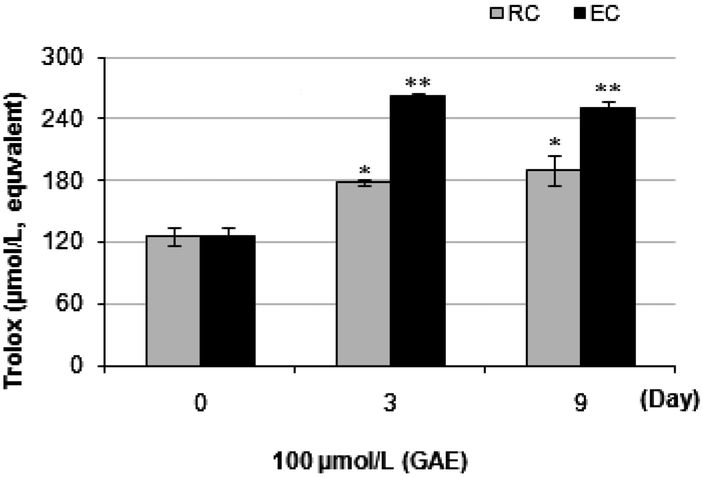

*Figure 6. Changes in antioxidant capacity (ORAC) of maqui berry juice during fermentation. RC and EC refer to maqui berry juice treatments inoculated with yeast RC and EC, respectively; Juice concentration was based the total polyphenol content described as GAE (gallic acid equivalents); Antioxidant capacity was determined using the ORAC assay described as Trolox equivalents (µmol/L). Data are expressed as means ± SD. *P < 0.05, ** P<0.01 compared to day 0.*

Similar to a report on blueberry juice (*37*), our study showed that fermentation with yeast increased the antioxidant capacity of maqui berry juice. Kahkonen et al. (*23*) suggested that fermentation was an even more effective method of extracting active phenolic compounds than solvent extraction, as the antioxidant phenolics could be concentrated during the wine-making process. This hypothesis was supported by our finding that, even though the TP content of the juices was tested at identical concentrations, the fermented maqui berry juice showed much higher ORAC value than the unfermented juice.

In previous reports, antioxidant activity of a berry extract in an LDL oxidation assay was related to the presence of anthocyanins, and positive correlation was also found between ORAC and anthocyanin content in berries (*33*). In our study on maqui berry juice fermentation, an initial increase at day 3 in anthocyanin content corresponded with an increase of ORAC values. However, after day 3, anthocyanin levels were diminished while ORAC values remained high. Similar observations were reported by Sanchez-Moreno et al. (*13*), who found no correlation between ORAC values and total anthocyanin content in both red (grape) wine and blueberry wine. The structure–antioxidant activity pattern emerging from studies suggests that potent antioxidant activity is associated with the presence of hydroxyl groups in the anthocyanin B-ring (*47*) . Previous reports have shown that wines with the highest contents of total polyphenols did not always show the highest values for antioxidant capacity, and that antioxidant capacity of wine may be more related to the type of phenolic compounds present (*48*). Additionally, Frankel (*49*) indicated that synergy between antioxidant compounds may have a significant effect on the antioxidant response. Synergistic interactions between components of the fermented maqui berry juice may partially explain the elevated ORAC values in our study. Therefore, the elevation of maqui berry juice ORAC after fermentation could be attributed to the decrease in sugar and the increase in gallic acid, the changes of tannins, the anthocyanin components profiles or the overall potentiating interactions between components.

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Inhibition of COX-2 and iNOS Protein Expression

The fermented and control juices had no visible effects on cell cytotoxicity at any concentration or at any time during the fermentation (data not shown). This indicated that both the unfermented and fermented maqui berry juices are safe for use with macrophages. In this study, the Griess Reagent assay was not applicable for NO determination because of interference of the colored maqui berry juice at the target wavelength. Since the production of NO is controlled by the inducible form of nitric oxide synthase, we studied the protein expression of iNOS in juice-treated RAW 264.7 cells using a Western blot method.

The EC fermented juice inhibited iNOS protein expression significantly more than unfermented juice (Figure 7B). However, unfermented juice (Day 0) was still capable of inhibiting iNOS protein expression at all concentrations tested. These results were similar to the inhibitory effect on NO production where fermented juice also had a stronger inhibition effect than unfermented juice at the same concentration (data not shown), whereas a dose-dependent inhibition was not observed in the (unfermented) maqui juice concentration tested in this study. To further understand the potential role of maqui berry juice in inflammation, we measured the protein expression of COX-2 on LPS-stimulated RAW 264.7 cells. The unfermented maqui juice at high concentrations of 200 and 400 μmol/L of GAE showed significant inhibition on protein expression of COX-2. Even at a low concentration, COX-2 was effectively inhibited (Figure 7C). However,

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COX-2 was effectively inhibited in LPS-stimulated macrophages treated with fermented juice at significantly lower concentrations (below 200 µmol/L GAE) than for unfermented juice ($P < 0.05$) (Figure 7D).

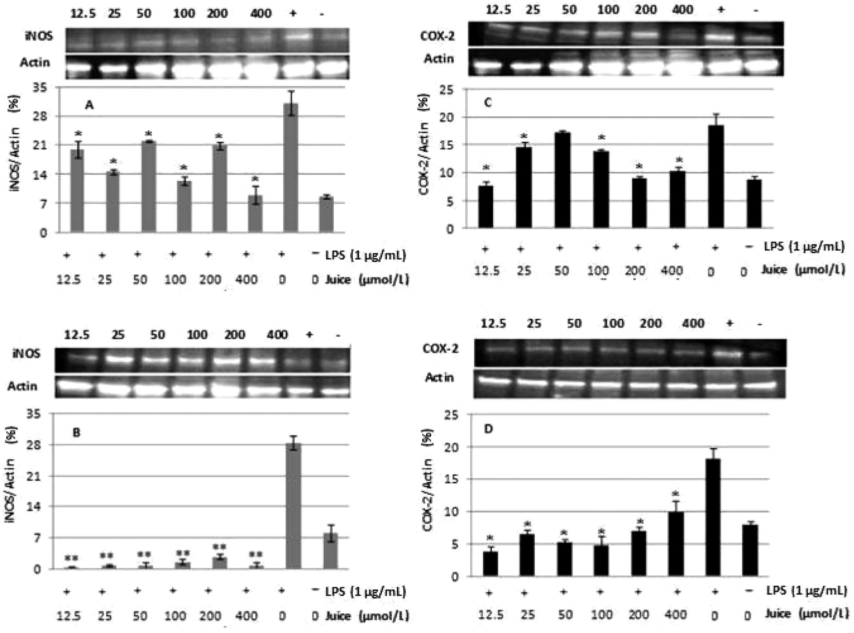

*Figure 7. The inhibitiory effects of maqui berry juice on protien expression of iNOS and COX- in LPS stimulated macrophages. The iNOS, COX-2 and actin level was measured by Western blot. The histogram represents quantification of the lanes of gels using a Kodak Image Station 440 CF. The 12.5, 25, 50, 100, 200, 400 labels identify macrophage cells treated with 1 µg/mL LPS and the corresponding juices (total polyphenol concentration described as gallic acid equivalents, GAE). A, C = unfermented maqui juice. B, D = EC fermentation treatment on day 9. Data are expressed as means ± SD. *P < 0.05, ** P<0.01 compared to LPS alone (+).*

In a recent study using LPS-activated macrophages and flavonoids from different classes, a strong correlation was found between the inhibition of iNOS expression and the interference with NF-kB pathway (*50*). Flavonoids have previously been shown to effect iNOS and COX-2 protein expression through mechanisms interfering with NF-kB activation in liver cells, and the most active compounds were quercetin, apigenin, luteolin and diosmetin, which downregulated iNOS expression more efficiently than kaempferol and chrysin. These observations corroborate previous assumptions about the structure-activity relationships, in that the $C_2 - C_3$ double bond plays a determinant role in the iNOS inhibition (*51*). Additionally, Comalada et al. (*50*) considered that the position of the B-ring is important to the level of activity, and the inhibitory effect is improved when the molecule has a high number of hydroxyl substituents.

COX-2 is highly expressed in the inflammation-related cell types including macrophages and mast cells when stimulated by LPS, phorbol esters, cytokines, or growth factors. This isoform is associated with acute and chronic inflammatory disorders. Although the role of flavonoids on COX-2 inhibition has not been conclusively determined, evidence supporting a suppressive effect on COX-2 expression by this group of compounds is building. Several reports suggest that proanthocyanidins, anthocyanins, and certain berry extracts can inhibit COX activity (*8*, *10*, *43*, *52*). For example, 40 μM anthocyanidins inhibited the activity of purified COX enzyme preparations in a cell-free system, and cyanidin was able to decrease COX-2 activity by 74% (*52*). Delphinidin inhibited LPS-induced expression of COX-2 mRNA and protein in mouse macrophage RAW264 cells. The ability of delphinidin to downregulate COX-2 expression may have been, at least in part, a consequence of its suppression of LPS-induced activation of NF-kB (*53*).

In the present study, maqui berry was much richer in anthocyanins (delphinidin, cyanidin and their glucose derivatives) than levels reported for wild blueberry, raspberry, chokeberry and strawberry. After fermentation, the decrease of delphinidin 3, 5-diglucoside and delphinidin 3-glucoside indicated possible release of the associated sugars. In tart cherries, the aglycones of anthocyanins (cyanidin, for example) were more efficacious than the glycosides, suggesting that the antioxidant activity of anthocyanins was due to their aglycone moiety (*54*). The number of sugar residues at the C3 position also seemed to be very important for antioxidant activity; anthocyanins that contained three sugar residues showed higher antioxidant activity than anthocyanins with one sugar residue.

The strong inhibition of the protein expression of iNOS and COX-2 of fermented juice might be attributed to: (1) an increase in gallic acid content in the fermented juice. (2) biotransformation of ANC and PAC during the fermentation by yeast, which might improve the bioactivity of the juices; (3) changes in the ratio of anthocyanins as delphinidin 3-sambubioside-5-glucoide, delphinidin 3, 5-diglucoside and delphinidin 3-glucoside and possible release of higher bioactivity moieties, and (4) potentiating interactions between antioxidant/anti-inflammatory compounds.

Conclusions

The main organic acid constituents of maqui berry were identified for the first time as quinic, citric, malic, chlorogenic, succinic and gallic acid. Most of the total sugar was removed by 3-day fermentation with yeasts. Fermented maqui berry juice had an elevated, potent antioxidant and anti-inflammatory activity as shown by inhibition of COX-2 and iNOS pathways in a macrophage cell line. Anthocyanin content changed dramatically during fermentation, with an increase in delphinidin 3-sambubioside-5-glucoside and delphinidin 3-sambubioside, and a decrease in delphinidin 3,5-diglucoside and delphinidin 3-glucoside. This study

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suggested that fermentation of maqui berry juice using yeast could be a feasible method to remove sugars for large scale production of maqui berry extracts with improved bioactivity while avoiding the use of potentially hazardous organic solvents.

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Abbreviations: COX-2: cyclooxygenase; NO: nitric oxide; iNOS:inducible NO synthases; SPE: solid-phase extraction; LDL: Low-density lipoprotein; TP: total polyphenol; GAE: gallic acid equivalents; PAC: proanthocyanidins; ANC: anthocyanins; C3G: cyanidin-3-*O*-glucoside; ORAC: oxygen radical absorbance capacity assay; TBST: Tris-buffered saline with Tween 20; LPS: lipopolysaccharide

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Chapter 7

Potential Therapeutic Applications of Common Agro-Food Byproducts and Chilean Wild Plants

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Procyanidins from grapeseed, grape pomace and pine bark were obtained by extraction with ethanol and further solvent partitioning to obtain a fraction soluble in water and both ethyl acetate, or directly recovered from Amberlite XAD16. Then, proanthocyanidin-enriched fractions were obtained by gel filtration through Sephadex LH-20 or Toyopearl HW-40F. Comparison of galloylated and non-galloylated procyanidins showed the influence of galloylation on the induction of apoptosis in colon cancer cells and in melanoma cells. Fractions from grapes showing galloylation percentages between 15 and 34 % and polymerization degrees between 1.7 and 3.4 induced apoptosis. The cardioprotective potential of proanthocyanidin-containing fractions from grape pomace was studied by measuring the inhibition of human endothelial NADPH oxidase. IC_{50} values for NADPH inhibition were in the range 2.5-3.4 mg/L. Extracts from green parts of murta

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

(*Ugni molinae Turcz.*) and maqui (*Aristotelia chilensis*) are rich in flavonols (kaempferol, myricetin, rutin,- among others) and the fruits rich in anthocyanins. Murta and maqui extracts showed anti-hemolytic activity and inhibited alpha-amylase, although not alpha-glucosidase.

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Introduction

Experimental results of bioactivity supporting the potential therapeutic use of fractions obtained from agro-food byproducts are shown and discussed in this chapter. The characteristic of raw materials noted as “common byproducts” is that they are widely spread out around the world. They are composed of flavan-3-ol oligomers (proanthocyanidins), either galloylated (grapeseed) or non-galloylated (pine). Extracts from the plants endemic from the South Chile murta (*Ugni Molinae Turcz.*) and maqui (*Aristotelia chilensis*) were also analyzed and determined to be composed mostly of flavonol derivatives (glucosides, xylosides, etc.), with minor amounts of proanthocyanidins. It can be seen in Figure 1 a scheme of the materials and performed assays.

Proanthocyanidin fractions from grapeseed and grape pomace have been studied by several authors, being their anti-carcinogenic and cardioprotective activity well established by *in vitro* and mice assays (*1–4*).

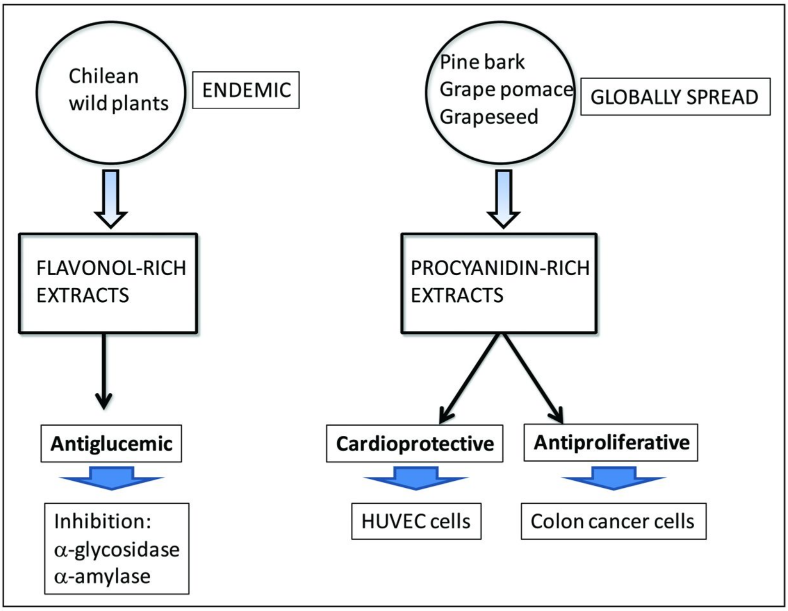
Chilean wild plants
ENDEMIC
Pine bark
Grape pomace
Grapeseed
GLOBALLY SPREAD
FLAVONOL-RICH EXTRACTS
PROCYANIDIN-RICH EXTRACTS
Antiglucemic
Cardioprotective
Antiproliferative
Inhibition:
α-glycosidase
α-amylase
HUVEC cells
Colon cancer cells

Figure 1. Schematics of materials and activities covered in this chapter.

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We present in this chapter some results on anticarcinogenic activity of galloylated and non-galloylated fractions obtained from grapeseed/grape pomace and pine bark, respectively.

The results of the research on extracts from chilean plants are more exploratory, aimed to analyze their potential usefulness for obtaining bioactive compounds. The polyphenolic profile, where flavonols were the major compounds (*5*), suggested that antioxidant and anti-obesity activities could be those more remarkable. Their antioxidant activity and capacity to inhibit α-amylase and α-glucosidase was determined by *in vitro* assays.

Because proanthocyanidin fractions from pine bark does not contain gallates, differently from tea or grape proanthocyanidins, the comparison of both types of compounds revealed the influence of galloylation degree on the antioxidant activity (*6*) and the antiproliferative activity. Galloylation has been also recently reported to play an important role in the anticarcinogenic activity of proanthocyanidins on prostate cancer (*3*).

Bioavailability of chemical substances taken from natural sources is a key aspect that determines their biological activity in human organism, either antioxidant, cardioprotective or anticarcinogenic. Foods or food complements containing these compounds are those called nutraceuticals, group within which is interesting to place the fractions from natural products with proven bioavailability, activity and safety. Several authors suggested that the metabolites of food polyphenols (*7–9*) were responsible for the biological activity, rather than the action of the "natural" compounds.

It has been reported that grapeseed or grape pomace proanthocyanidins that showed remarkable *in vitro* antioxidant and apoptotic-induction activities were those fractions of mean polymerization degrees within the range 2-4. (*10*). Consequently, these compounds are not likely to be readily absorbed because of their high size, thus reaching colon in almost intact form, where the interaction with colon microorganisms is the factor determining their chemical modifications. For this reason, assays on colon cancer cells were among the first in vitro assays reported the anticarcinogenic activity of flavonoids, together with the commercial availability of Caco-2 cells (nowadays many other cancer cell lines are being studied). This topic has been studied for monomers, but not for galloylated proanthocyanidin dimers, trimers and higher oligomers.

In vivo human studies regarding the action of flavan-3-ols or proanthocyanidins are scarce and performed mainly on galloylated monomers, as epigallocatechin gallate from tea, which has also been extensively studied *in vivo* (*1*, *4*). Vasorelaxing and blood antioxidant activities have been confirmed in human studies for grapeseed proanthocyanidins (*11*, *12*)), so as the effect on the oxidative stress (*13*).

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Evaluation of Grape and Pine Bark Proanthocyanidin-Containing Fractions for Cancer Therapy

The activity of fractions from grape pomace and pine bark was assayed for apoptosis induction in colon cancer cells. The influence of galloylation was analyzed by assaying galloylated (from grapeseed or grape pomace extracts) and non-galloylated procyanidins (from pine bark).

Methodology

Proanthocyanidin-enriched fractions were obtained from OW fraction (a fraction which is soluble in both water and ethyl acetate), described by Torres et al. (*14*). Gel chromatography of this fraction through Toyopearl HW-40F and Sephadex LH-20 rendered several fractions with different polymerization degrees and also different galloylation percentages when grapes were the substrate. A general procedure for performing this elution was reported by Touriño *et al.* (*15*). The mean degree of polymerization (mDP) and the percentage of galloylation (%G) were determined according to Torres et al. (*14*).

The influence on cell cycle was performed by flow cytometry according to the methodology reported by Lizárraga *et a*l. (2007) (*2*). Antiproliferative activity was measured as IC_{50}, that is the concentration values of procyanidin-enriched fraction that achieve a reduction of cell growth by 50%.

Results and Discussion

Apoptosis assays were performed at IC_{50} dose, being the results shown in Table I.

Table I. Apoptosis induction by grape pomace and pine bark fractions

	mDP	*% Galloylation*	*% EA apoptosis at IC_{50}*	*% LA apoptosis at IC_{50}*
Grape	3.4	34	18	7
	2.7	25	11	5
	2.4	16	4	3
Pine bark	3.4	0	--	
	3.0	0	10	7

EA: Early apoptosis, LA: Late Apoptosis. mDP: mean degree of polymerization

Proanthocyanidin oligomers influenced the early apoptosis rather than the other stages, as is shown in Table I. Fractions from pine bark (not containing gallates) and showing mDP values about 3 promoted a slight degree of apoptosis (10%), but other fractions from pine were inactive.

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All of grape fractions showing galloylation percentages between 16 and 34 % and mDP values between 2.4 and 3.4 induced apoptosis, the activity increasing with mDP value.

The importance of galloylation in hydroxyl scavenging activity as measured by electronic spin resonance was determined by Lizárraga *et al.* (*16*), reporting that a fraction from white grape pomace with mDP value 3.4 and 34% galloylation showed an scavenging activity almost twice than similar ones from pine bark (mDP=2.9) at 50 mM concentration: 70% for grape vs 45% for pine bark.

In a recent study, Chou et al. (*3*) reported that galloylation degree influenced the induction of caspase-dependent apoptotic death in prostate carcinoma LNCaP Cells by proanthocyanidins, being di-galloylated dimer the most active. Similarly to the results shown in this work, they also found no activity of non-galloylated proanthocyanidins.

They observed that for mono-galloylated dimers, the position of the gallate ester link also influenced the activity, being it higher when the gallate was linked to 3′-moiety (in the terminal unit) than when it was attached to 3-moiety. This result suggests that the feasibility of a direct interaction between the gallate moiety and biomolecules is an important factor in the activity of gallates. The mechanism explaining these differences on the anticarcinogenic action on several cell classes remains unknown till date, apart from the mechanisms merely related with the antioxidant action on radicals.

Not much nutrition or epidemiological studies have been developed in humans to assess the anticarcinogenic activity of flavan-3-ol derivatives, none of them specifically reporting data about the intake of galloylated-proanthocyanidin oligomers. In a recent epidemiological study, Cutler *et al.* (*17*) reported that the intake of flavonoids diminished the risk of lung cancer in post-menopausical women, which was regarded with the past as smoker of the individuals and, interestingly, the positive effect was not significant for non-smokers. A limitation of this study is that several flavonoid families were not correlated, being data based in nutritional tables. As they pointed out, there was an inconsistence in epidemiological studies results, due to the use of different nutritional tables and the lack of complete flavonoids profile. Additionally, recent studies in mice confirmed the anti-inflammatory and anticarcinogenic potential of grapeseed extracts in vivo (*18*, *19*).

Thus, the combination of antioxidant and cardioprotective activities of proanthocyanidins, either galloylated or not, together with the anticarcinogenic activity of the galloylated ones, points out grapeseed or grape pomace fractions to be among the most interesting for obtaining bioactive compounds.

Evaluation of Grape and Pine Bark Proanthocyanidin-Containing Fractions as Potential Cardioprotective Agents

Production of reactive oxygen species (ROS) has been related with atherosclerosis, hypertension, vasculopathy and restenosis. Superoxide radical is the main compound of this class in vascular endothelium, being the action of NADPH oxidase its main source (*20*). The capacity of proanthocyanidins from grape to inhibit NADPH in cells oxidase was studied as an indicator of potential cardioprotective activity.

Methodology

NADPH inhibition activity was analyzed on Human Umbilical Vein Endothelial Cells (HUVEC). HUVEC were placed at a concentration of 10,000 cells per well in 96-well microplates with supplemental endothelial cells. Serum and supplements were subjected to a starvation period of 12 h before adding fractions indicated in Table II. NADH oxidase inhibition in HUVEC lysates was performed using lucigenin-enhanced chemiluminiscence (*21*). Superoxide scavenging activity was determined in the system phenazine methosulfate (PMS-NADH) and quantified by reduction of nitro blue tetrazolium (NBT), as reported by Alvarez et al. (*22*) with minor modifications regarding the specific assay with grape fractions: 600 µl test solutions were made up in a phosphate buffer (50 mM KH2PO4-KOH, pH 7.4) containing 166 µM β-nicotinamide adenine dinucleotide (NADH, Sigma-Aldrich), 43 µM NBT, and SOD (10 U/ml) in the absence or in the presence of fractions selected fractions by their polymerization and galloylation degrees (F4, F5 or F6 in Table II) at concentrations ranging 1 – 10 µg/mL.

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Results and Discussion

The inhibition of human endothelial NADPH oxidase was analyzed *in vitro* for three fractions with galloylation percentages in the range 14 to 25% and mean polymerization degrees of 2.5, 3.8 and 5.3. IC_{50} values for NADPH inhibition test were obtained in the range 2.5-3.4 mg/L (Table II). Taking into account the theoretical molecular weight of these galloylated proanthocyanidin oligomers, these values should be in the micromolar range (from 1.7 to 5 µM). These IC_{50} values are the same order of magnitude than those of flavonoids that have been reported to be strong NADPH-oxidase inhibitors, such as isorhamnetin, reported by Steffen *et al.* (*23*), or fatty-acid-synthase/NADPH inhibitors such as morin, reported by Li *et al.* (*24*).

Active compounds in the fractions demonstrated to be cell permeable and act at level similar to that of other inhibitors as apocynin or diphenyleneiodonium (DPI), with the advantage of being more hydrophilic and totally soluble in water at the active concentrations.

These results show the capacity of these fraction to inhibit NADPH oxidase, likely it being one of the modes of action that explains their cardioprotective action, which is suggested by epidemiological studies in humans, although not conclusively (*25*). The reduction of plasma oxidative stress after ingestion of grapeseed proanthocyanidins was confirmed by *in vivo* asays in humans, although this study was not addressed to elucidate the reduction of hypercholesterolemia or risk of cardiac events (*13*).

In contrast, the anticarcinogenic activity reported by *in vitro* (*2*, *3*) and *in vivo* assays in mice or rats (*1*, *26*) has not been clearly confirmed in epidemiological

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studies developed in recent years (*27*); so it is an important challenge of clinical nutrition research.

Table II. NADPH oxidase inhibition and scavenging activity of grape procyanidin-enriched fractions

Fraction	*mDP*	*% Galloylation*	*IC_{50} values for NADPH oxidase inhibition test (mg/L)*	*Superoxide scavenging in PMS-NADH system (mg/L)*
F4	2.5	14	3.4	21.7
F5	3.8	20	3	5.5
F6	5.3	25	2.5	12

mDP: mean degree of polymerization

Proanthocyanidins have demonstrated important activity on plasma oxidative level in rats (*13*, *28*), although anthocyanins (*29*, *30*) are the most extensively studied compounds in humans till date. Both types show a limited bioavailability, being found in plasma metabolized as glucuronates (*12*, *31*), lactones (*7*) or aglicones (*29*).

Assays in mice have demonstrated the blood-pressure reducing activity of non-galloylated procyanidins, due to the increase of phosphorylated-endothelial nitric oxide synthase (Ser1177) expression in aortic tissues (*26*), although authors did not identify which procyanidins could be bioavailable and then being responsible for the action, because a commercial extract was used.

Those oligomeric fractions containing between 3 and 4 monomers (as mean value), which are in theory not available by oral intake, have been found to be the most active on cells. Consequently, it is not likely that consumption of these compounds in diet should reproduce the results of *in vitro* assays. It is clear that clinical studies in humans using well-defined proanthocyanidin fractions are necessary, because *in vivo* assays with mice support some biovailability of proanthocyanidins. The study of metabolites from proanthocyanidin oligomers is also nowadays a challenge and interesting studies addressed to determine the metabolites of flavonoids in pigs (*32*) are being developed in this area.

Grape proanthocyanidins have also shown some anti-inflammatory activity. In a recent study, Terra *et al.* (*33*) have studied the anti-inflammatory effects of grapeseed proanthocyanidins in rats, reporting that a statistically significant increase of adiponenctins expression was found *in vivo*, together with a reduction of the inflammation markers TNF-α, interleukin-6 and C-reactive protein and also it was reduced the NF-kB activity.

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Applications as Antiglycemic Agents of Extracts from Flavonol-Enriched Extracts

In our search for new vegetal species to obtain bioactive compounds, several species of wild berries grown in Araucania (Chile) were considered as an interesting source. The studied plants were murta (*Ugni molinae Turcz.*) and maqui (*Aristotelia chilensis*). Murta berries and leaves have been consumed by the mapuches Maqui grows in the wild, but also it is being cultivated nowadays for obtaining its fruit, very rich in anthocyanins. Their leaves showed an interesting profile because of the content in procyanidins and flavanol derivatives (*5*, *34*).

Methodology

Ethanol extracts from murta and maqui grown in the Araucania region (Chile) were obtained in the conditions reported by Rubilar *et al.* (*5*). These materials were processed for obtaining of OW fraction as described by Torres et al., (*10*). Alpha-amylase inhibition assay was performed using the chromogenic method adopted from Sigma–Aldrich, using porcine pancreatic α-amylase at 4 UI/mL. Solution of plant extracts (200 µL) and 400 µL of starch solution were mixed and preincubated for 5 min. Addition of 200 µL enzyme solution started the reaction, which was stopped by addition of dinitrosalicylic acid (DNS) reagent after 15 minutes to measure the released reducing sugars.

Alpha-glucosidase analysis was performed using p-nitrophenyl-α-D-glucopyranoside (pNPG) as substrate. 50 µL of α-glucosidase (0.6 U/mL) was premixed with the plant extract (50 µL) in 67 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.8). This mixture was preincubated at 37 °C for 10 min and the reaction started with the addition of 125 µL of 10 mM pNPG and incubating for 30 min. p-nitrophenol was measured at 400 nm.

Results and Discussion

Most of the oligomeric proanthocyanidins concentrated in OW fraction, although some of them remained in the aqueous phase. HPLC chromatograms showed a similar profile for OW fractions and for the initial ethanol extract, because of the low content in oligomeric proanthocyanidins in the green parts of these plants, as compared with the monomeric flavanol derivatives, which were those more abundant and reported to be the responsible for the astringency.

Ethanol extracts from the green parts of these plants are rich in flavonols, being rutin the major compound in murta, which also contained kaempferol and myricetin, among others (*34*). It has also been reported the antimicrobial activity of extracts from this plant, that was related to the concentration of flavanol glucosides (*34*).

Fruits from murta and maqui are very rich in anthocyanins, being malvidin triglucoside and cyanidin cumaroilglucoside identified (data not published), but a complete profile has not been reported yet. Both anthocyanins and flavonols have shown *in vitro* antioxidant activity and also the cardioprotective activity of berry anthocyanins (*29*) has been reported in several studies performed in the last decade (*35*).

The interest on the study of green parts of these plants relies on the presence of flavonols, since the antiglycemic activity of these phytochemicals has been reported (*36*).

Table III. Alpha-amylase inhibition by murta and maqui extracts

		IC_{50} α-amylase inhibition (mg/L)		
		*Raw extract**	*OW fraction*	*Aqueous fraction*
Murta	*Leaf*	79.5 ± 3.1	165 ± 30.2	110.1 ± 1.9
	Stems	56.6 ± 1.2	n.d.	--
Maqui	*Leaf*		521.5 ± 7.6	314.2 ± 2.9
	Fruit	41.5 ± 3.6	n.d.	--
Acarbose		3.4		

--:Missing data, meaning that yield was too low to assay this fraction n.d: not detected activity higher than 50%

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Inhibition of α-amylase and α-glucosidase results are shown in Tables III and IV. The extracts showed low level of alpha-amylase inhibition as compared with acarbose, which is a specific inhibitor. Raw extract from murta stems maqui fruit were those with a slight inhibition of this enzyme. Low molecular weight of the flavonols can explain the low efficiency in blocking α-amylase active center, differently from acarbose. The measurable but low inhibiting activity of leaf extracts might be explained by procyanidin oligomers present at low concentration.

In contrast with the assays on α-amylase, α-glucosidase was highly inhibited by extracts from murta and maqui leaves (Table IV). Except for maqui leaves, the separation process for obtaining OW fraction diminished the specific inhibiting activity, likely due to the loss of some active monomers.

Alpha-glucosidase activity in murta extracts can be attributed mainly to rutin, which α-glucosidase inhibition activity was reported by Li *et al.* (*37*), although kaempferol and myricetin glucosides in murta (see Table V) could also contribute to this action.

Metabolites from rutin as glioxal or 3,4-dihydroxyphenylacetic acid have been reported as powerful inhibitors of the histone H1 glication (*38*). Inhibition of glication is an additional mode of action, by which murta extracts may be helpful for the diabetes treatment.

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Table IV. Alphaglucosidase inhibition by murta and maqui extracts

		IC_{50} α-glucosidase inhibition (mg/L)		
		*Raw extract**	*OW fraction*	*Aqueous fraction*
Murta	*Leaf*	12.5 ± 1.8	215 ± 6.3	110.1 1.9
	Fruit	69.2 ± 5.0	61.3 ± 7	
	Stems	39.1 ± 1.2	n.a.	--
Maqui	*Leaf*	6.1 ± 0.9	2.4 ± 0.3	2.4 ± 0.3
	Fruit	47.9 ± 2.7	n.a.	n.a.
	Stems	1.1 ± 0.1	189.4 ± 27.7	112.3 ± 16.5
Acarbose		247		

n.a.: not detected, --: too low yield to isolate

As was expected, acarbose showed a low inhibiting activity, likely because of its specific design with high enough molecular weight for effectively blocking the active center of α-amylases; it cannot block α-glucosidases active center, which is naturally optimized to link molecules the size of disaccharides or, at most, trisaccharides.

Raw extracts from maqui leaves and stems showed the strongest α-glucosidase inhibition activity and, differently from murta extracts, the process applied for obtaining OW fraction did not dramatically reduce its activity. This result can be explained by the higher content of proanthocyanidins in maqui than in murta. The polyphenolic profile of ethanol raw extracts and OW fractions from maqui polyphenols is described in a paper by Rubilar *et al.* (*39*) being the major compounds indicated in Table V. Flavan-3-ol oligomers, quercetin glucosides and quecetin rhamnosides were the major compounds in raw extracts. Additionally, ion-positive mass fragments that were very similar to sodium adducts of galloylated procyanidins were detected and also a fragmentation pattern very similar to that of epicatechin gallate was observed. The identification of those peaks is being performed nowadays, being currently a work in progress.

Qualitative Composition of the Fractions or Extracts

The major flavonoids in proanthocyanidin-enriched fractions from pine bark and grape pomace, together with those in OW fractions and ethanol extracts from murta and maqui are listed in Table V. Although the analyzed bioactivities were not correlated with any particular molecule in the fractions, the overall list of compounds helps us to explain the observations, taking into account previous reported bioactivities. For example, the prevalence of galloylated proanthocyanidin oligomers in grapeseed and their lack in pine bark is related wit induction of apoptosis, or the presence of several flavonol glucosides in murta is likely related with α-glucosidase inhibition. We exclude here most of benzoic and cinnamic acid derivatives because, despite of existing at non-negligible levels in raw extracts, they are minor compounds in proanthocyanidin-enriched and OW fractions, so as in ethanol extracts from murta (Table V).

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Table V. Main polyphenols flavonoids of fractions obtained from aqueous and ethanol extracts from agro-food residues

White Grapeseed[1] *(Proanthocyanidin-enriched fractions from aqueous extract)*	Gallic acid Catechin Epicatechin Catechin gallate Epicatechingallate Di-galloylated dimer (P_2G_2) Procyanidin dimers (Cat-Cat and Ec-Cat) Procyanidin trimers. Other galloylated oligomers (P3G1, P3G2..) Quercetin glucoside
Pine bark[2] *(Proanthocyanidin-enriched fractions from ethanol extract)*	Gallic acid Catechin Epicatechin Dihydroquercetin[3] Piceatannol[3] Procyanidin B2 Procyanidin C1 Procyanidin oligomers Procyanidin trimers Oligomers, DP from 4 to 8 in *Pinus pinaster* and 4 to 11 in *Pinus radiata*.
Murta leaves[4] *(Ethanol extract and OW fraction)*	Epicatechin myricetin dirhamnoside myricetin glucoside quercetin dirhamnoside myricetin rhamnoside quercetin glucoside kaempferol glucoside quercetin rhamnoside
Maqui leaves[5] *(Ethanol extract)*	Protocatechuic acid. Catechin, Epicatechin Quercetin dirhamnoside Quercetin hexoside (glucoside/galactoside) Quercetin xyloside Quercitrin Non-identified oligomeric flavanols

[1] Data from Ref. (*40*, *41*) [2] Data from Ref. (*42*) [3] Data from Ref. (*43*) [4] Data from references (*5*) and (*34*) [5] Data from ref. (*39*)

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Conclusions

Pine bark and grape pomace or grapeseed are widely spread materials that render proanthocyanidin-enriched fractions. It has been observed the influence of galloylation on the antioxidant and antiproliferative activity of proanthocyanidin oligomers. Those fraction obtained from pine were much less active than those from grapeseed or grape pomace, although these have demonstrated their antioxidant antioxidant activity in blood and have been assayed *in vivo* with success. Bioavailability is a key factor that must be studied because those more antiproliferative fractions from grape are a size that indicates low or none availability by oral intake. Metabolites originated by the action of microorganisms in colon may promote more absorbable and active compounds or by the contrary, to produce inactive compounds. It is not likely that citotoxic compounds were formed, because it has been observed in general the benefits of consuming proanthocyanidins, but is necessary further research. Studies in humans with a strict size-defined proanthocyanidins are limited by the availability of fractions: till now there is no way of synthesis of these compounds and the commercial grapeseed extracts must be fractioned with a usually low yield, which also depends on the variety and previous grape processing.

Fractions obtained from murta (*Ugni Molinae Turcz.*) and maqui (*Aristotelia chilensis*) confirmed their capacity to inhibit α-glucosidase but not inhibiting α-amylases. Main flavonoid in murta is rutin which was previously reported to be a strong α-glucosidase inhibitor. These results address a potential use as a food complement to help in glycemia reduction, in combination with specific amylase inhibitors, which dosage might be reduced.

Profiling of aqueous extracts and *in vivo* studies are necessary to confirm the benefits of murta and extracts and also it is necessary to develop cytotoxic analysis to confirm their safety.

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Chapter 8

Isolation, Structure Elucidation, Synthesis, and Cytotoxic Activity of Polyacetylenes and Polyenes from *Echinacea pallida*

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This study focuses on presenting an overview of recent results on the cytotoxic activity of polyacetylenes and polyenes isolated from *Echinacea pallida*. In a search for biologically active compounds from plants of the genus *Echinacea*, the lipophilic extract from *E. pallida* roots was characterized by a higher cytotoxic activity if compared with the other tested species. A subsequent bioassay-guided fractionation allowed the isolation and structure elucidation of ten polyacetylenes and polyenes from *E. pallida* roots. The isolated secondary metabolites were tested for their cytotoxic activity on selected human cancer cell lines and (8Z,13Z)-pentadeca-8,13-dien-11-yn-2-one was the most active constituent, particularly on the colonic COLO320 cancer cells ($IC_{50} = 2.3 \pm 0.3$ μM) and breast carcinoma MCF-7 cancer cells ($IC_{50} = 2.5 \pm 0.7$ μM). Arrest of cell cycle in the G1 phase and induction of apoptosis were found to be involved in its mechanism of action. Due to the difficult purification of this compound from the plant material, its first total synthesis was also described. A HPLC stability study of this natural product finally indicated that its cytotoxic activity can be mainly attributed to the genuine, not oxidized, molecule. These results indicated that polyacetylenes and polyenes from *E.*

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pallida are effective in inhibiting cancer cell proliferation and incorporation of enriched fractions of these compounds in the diet may be useful for cancer prevention. The most active compound represents also an interesting lead structure for the development of new antiproliferative agents.

Introduction

In the ambit of the genus *Echinacea* (Asteraceae family), the species traditionally used in phytotherapy are *E. purpurea* (L.) Moench, *E. angustifolia* DC. var. *angustifolia* and *E. pallida* (Nutt.) Nutt. (*1*). The drug consists of the roots, but, in the case of *E. purpurea*, aerial parts are also employed. *Echinacea* extracts are commonly applied in the formulation of dietary supplements and herbal remedies. The main use of these natural products is as immunostimulants in the prevention and treatment of inflammatory and viral diseases (*1*).

Echinacea extracts showed a highly complex chemical composition, including: polar compounds (caffeic acid derivatives) (*2*, *3*), non-polar compounds (alkylamides and acetylenic secondary metabolites) (*4*, *5*) and high molecular weight constituents (polysaccharides and glycoproteins) (*6–8*). Alkylamides have been isolated from *E. purpurea* and *E. angustifolia* (*9*, *10*); very often, these secondary metabolites have one or more acetylenic groups in the terminal part of their chain. Many studies have shown the composition and the biological activity of alkylamides from *E. purpurea* and *E. angustifolia* (*11*). On the other hand, there is little research on the lipophilic secondary metabolites from *E. pallida*.

Acetylenic compounds of different sources have proven to be cytotoxic against different types of cancer cells and to enhance the cytotoxic activity of other anticancer drugs (*12*). In this ambit, this review will summarize the results of recent studies aimed at the evaluation of the cytotoxic activity of *Echinacea* root extracts and at the isolation and characterization of the bioactive secondary metabolites by means of a bioassay-guided fractionation.

The steps of the work can be summarized as follows:

- selection of plant material and preparation of crude extracts;
- primary assays;
- isolation and structure elucidation of secondary metabolites;
- secondary assays;
- structure-activity relationship;
- total synthesis of the most active compound;
- phytochemical analysis of *E. pallida* plant material and natural products;
- stability study.

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Selection of Plant Material and Preparation of Crude Plant Extracts

The whole study was focused on the lipophilic extracts from the three medicinally used *Echinacea* species (*E. purpurea*, *E. angustifolia* and *E. pallida*). The extraction was carried out by means of a conventional extraction method with a non-polar solvent (*13*). In particular, powdered dried *Echinacea* roots (10 g) were extracted with a Soxhlet apparatus for 4 h using *n*-hexane (200 mL). The extracts were evaporated to dryness under vacuum and dissolved in DMSO to obtain stock solutions at 150 mg/mL.

Primary Assays

The primary assays were carried out to evaluate the biological activity of the crude plant extracts on selected human cancer cell lines. In particular, the *Echinacea* lipophilic extracts were tested for their cytotoxic activity in primary assays on human pancreatic adenocarcinoma MIA PaCa-2 and human colorectal adenocarcinoma COLO320 cell lines in the concentration range 1-300 µg/mL (*13*). The attention was focused on these cancer types since pancreatic and colo-rectal cancers represent the fourth and the second leading causes of cancer death for both men and women in the United States (*13*). Cell viability was assessed by using the cell proliferation reagent WST-1, which is based on the cleavage of the tetrazolium salt 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate (WST-1) to formazan by mithocondrial dehydrogenase activity. Inhibition of cell proliferation was evaluated as the percentage reduction of UV absorbance at 450 nm of treated cells *vs.* control cultures.

The results of the primary assays indicated that *n*-hexane root extracts from the three tested *Echinacea* species exhibited a significant concentration-dependent decrease in MIA PaCa-2 and COLO320 cell viability after 72 h exposure in the concentration range 1-300 µg/mL (*13*). Figure 1 shows the concentration-dependent antiproliferative effect of *Echinacea* root extracts: the first graph is related to the cytotoxic activity on the pancreatic cancer cell line and the second on the colonic cancer cells.

The IC_{50} values of the three *Echinacea* species were then calculated and data are shown in Table I (*13*): *E. pallida* extract showed a higher cytotoxic activity if compared with *E. purpurea* and *E. angustifolia*. The cytotoxic effect of *Echinacea* extracts was more pronounced towards COLO320 than MIA PaCa-2 cells: in the case of *E. pallida* root extract, the IC_{50} value was 10.6 ± 0.7 and 46.4 ± 0.9 µg/mL in COLO320 and MIA PaCa-2 cancer cells, respectively (*13*).

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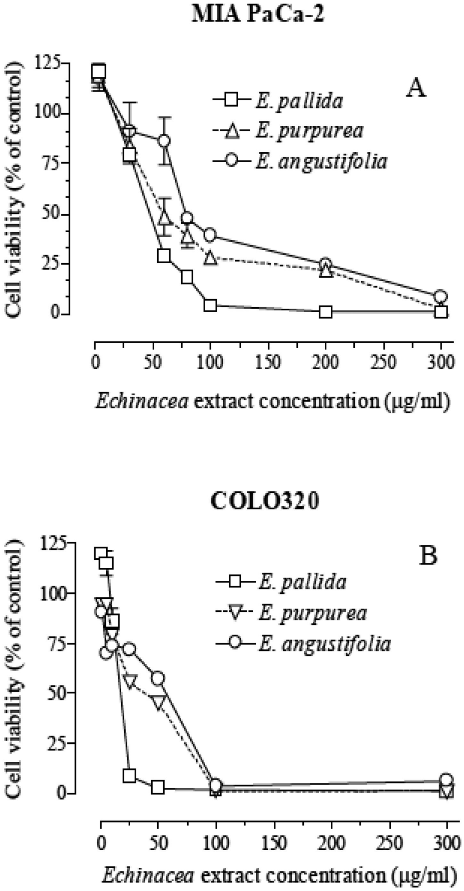

Figure 1. Concentration-dependent cytotoxic activity of Echinacea lipophilic extracts on MIA PaCa-2 (A) and COLO320 (B) cancer cell lines after 72 h exposure in the concentration range 1-300 µg/mL. Reproduced with permission from reference (13). Copyright 2006 Elsevier.

Further experiments were carried out to characterize the time-dependent antiproliferative effect of *E. pallida* extract, by expanding the time-exposure evaluation from 4 to 72 h at 25-100 µg/mL (*13*). Figure 2 shows the results obtained on the pancreatic and colonic cancer cell lines, respectively (*13*). At tested concentrations, a decrease in cell viability was observed by increasing the exposure-time, indicating a time-dependent antiproliferative effect for *E. pallida* root extract.

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Table I. Effect on cell viability (IC_{50}: inhibitory concentration at 50% effect level) on MIA PaCa-2 and COLO320 cells by *Echinacea* species[a]. Source: Reproduced with permission from reference (*13*). Copyright 2006 Elsevier

	IC_{50} (μg/mL), mean ± S.E.	
	MIA PaCa-2[b]	*COLO-320[b]*
Echinacea pallida	46.41 ± 0.87	10.55 ± 0.70
Echinacea purpurea	62.92 ± 2.24	25.36 ± 1.14
Echinacea angustifolia	82.86 ± 1.47	31.78 ± 1.99

[a] Changes in the IC_{50} mean values obtained for each species in MIA PaCa-2 *vs.* COLO320 cells are significant ($P < 0.05$; Student's *t*-test). [b] Within-species variations in the IC_{50} mean values are significant ($P < 0.05$; ANOVA followed by the Newman–Keuls test).

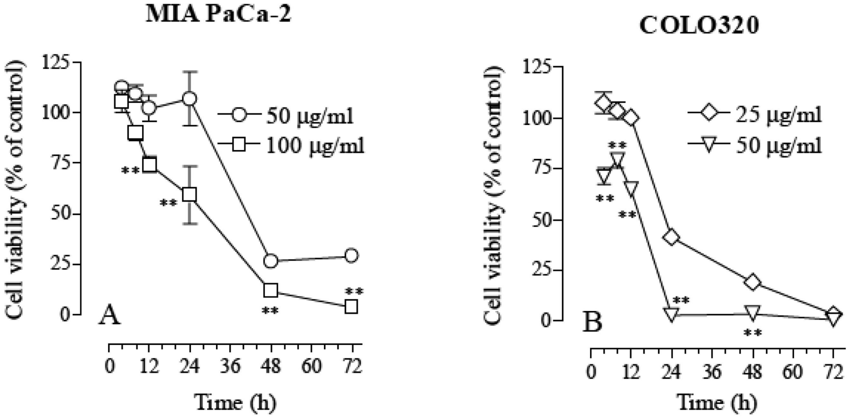

Figure 2. Time-dependent cytotoxic activity of E. pallida lipophilic extract on MIA PaCa-2 (A) and COLO320 (B) cancer cell lines. Reproduced with permission from reference (13). Copyright 2006 Elsevier.

To evaluate whether the cytotoxic effect could be dependent on the activation of caspases 3/7, two enzymes involved in the induction and execution of apoptosis, the *E. pallida* extract was tested for cytotoxic activity on MIA PaCa-2 and COLO320 cells at 100 and 50 μg/mL for 24 h, respectively (*13*). Apoptosis is a cellular mechanism of self-destruction, involved in physiological as well as pathological processes and it can be either spontaneous or induced by exogenous agents among which are many drugs already used in cancer therapy. Figure 3 shows the results of the caspase 3/7 activity assay and the DNA fragmentation assay, expressed as fold stimulation and enrichment factor of nucleosomal fragments *vs.* untreated cells, respectively (*13*).

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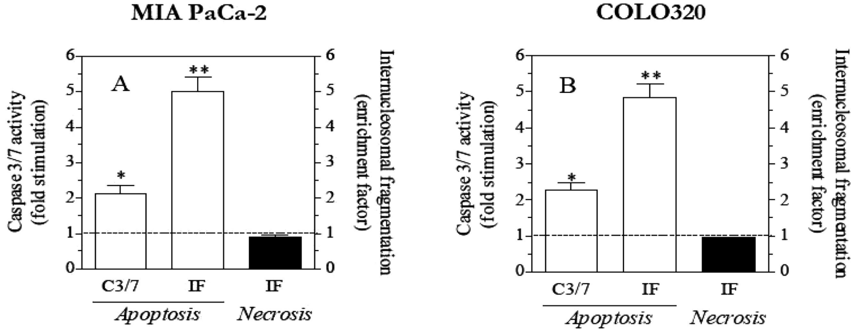

Figure 3. Apoptotic and necrotic cell death after treatment with E. pallida lipophilic extract for 24 h on MIA PaCa-2 (100 µg/mL) and COLO320 (50 µg/mL) cancer cell lines. Reproduced with permission from reference (13). Copyright 2006 Elsevier.

At tested concentrations, *E. pallida* extract produced a 2-fold increase in the caspase 3/7 activity in both cancer cell lines, which was paralleled cy a 5-fold cytosolic enrichment of apoptotic nucleosomes in both cases (*13*). Necrotic cell death was not observed in both cancer cell lines. This suggests that apoptotic cell death may account, at least in part, for the cytotoxic activity of *E. pallida* extract, and it may occur via a caspase-dependent mechanism.

Isolation and Structure Elucidation of Secondary Metabolites

According to U.S. National Cancer Institute (NCI) guidelines, the criteria of cytotoxic activity for crude extracts of an IC_{50} value below 30 µg/mL legitimated further investigations on single active constituents from *E. pallida*, the most active species. The lipophilic extract of *E. pallida* roots was therefore subjected to a bioassay-guided fractionation to identify the secondary metabolites responsible for the cytotoxic activity of the crude plant extract (*14*, *15*). After the extraction of the plant material with *n*-hexane, the isolation of the secondary metabolites was performed by a series of normal- and reversed-phase flash column chromatography (*14*, *15*). The subsequent structure elucidation of the isolated compounds was carried out by spectroscopic and spectrometric methods (*14*, *15*). The degree of purity of the isolated compounds was checked by HPLC and it was found to be about 98%.

Figure 4 shows the molecular structures of the compounds isolated from *E. pallida* roots (*14*, *15*): they include three hydroxylated acetylenes (**1**-**3**), two dicarbonylic acetylenes (**4**,**5**), three monocarbonylic acetylenes (**6**-**8**) and one monocarbonylic alkene (**9**). The structures of the secondary metabolites were determined on the basis of UV, IR, NMR (including 1D and 2D experiments, such as COSY, HSQS, HMBC and NOESY) and MS data (*14*, *15*). The structures of compounds **2** and **3** were confirmed by comparison with the literature (*16*).

Table II shows the ^{1}H-NMR data of the isolated compounds (*14*, *15*). The NMR data include the chemical shifts (ppm) and the coupling constants (Hz) obtained in $CDCl_3$. The ^{13}C-NMR data of the compounds of interest are shown in Table III (*14*, *15*).

As shown in Figure 5, the RP-HPLC analysis of *E. pallida* root extract showed the presence of two further peaks, in the region of more lipophilic secondary metabolites (*17*). A preliminary NMR and MS analysis of purified fractions indicated that the first one, eluting at 20 min, was actually a mixture of at least three compounds and was not further pursued, while the second one, eluting at 23 min, was purified as a single metabolite (*17*). However, due to the overlap of both olefinic and allylic proton resonances of the latter compound, it was not possible to exactly assign the configuration of the double bond between C-11 and C-12 by NMR data.

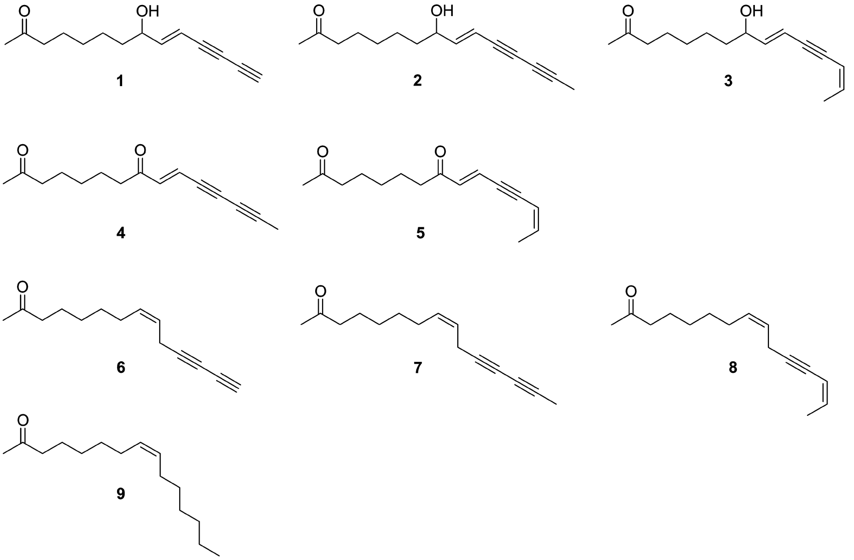

Figure 4. Chemical structures of polyacetylenes and polyenes isolated from E. pallida roots.

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Table II. ^{1}H NMR spectral data [δ (ppm), *m*, *J* (Hz)] of compounds 1–9 (400 MHz, CDCl$_3$, TMS as reference). Source: Adapted with permission from references (*14*, *15*). Copyright 2006 Elsevier

Position	***1***	***2***	***3***	***4***	***5***	***6***	***8***	***9***
1	2.17 *s*	2.17 *s*	2.17 *s*	2.16 *s*	2.16 *s*	2.16 *s*	2.15 *s*	2.14 *s*
2								
3	2.48 *t* (7.3)	2.46 *t* (7.5)	2.46 *t* (7.2)	2.46 *t* (7.3)	2.46 *t* (7.4)	2.46 *t* (7.3)	2.44 *t* (7.4)	2.43 *t* (7.4)
4	1.62 *m*	1.60 *m*[a]	1.62 *m*[a]	1.61 *m*[a]	1.62 *m*[a]	1.61 *m*	1.60 *m*	1.59 *m*
5	1.36 *m*	1.34 *m*[a]	1.34 *m*[a]	1.33 *m*	1.35 *m*	1.33 *m*	1.32 *m*	1.29 *m*[a]
6	1.42 *m*	1.34 *m*[a]	1.34 *m*[a]	1.65 *m*[a]	1.64 *m*[a]	1.42 *m*	1.40 *m*	1.34 *m*[a]
7	1.57 *m*	1.65 *m*[a]	1.63 *m*[a]	2.56 *t* (7.3)	2.58 *t* (7.5)	2.07 *m*	2.09 *m*	2.02 *m*
8	4.24 *dd* (5.2, 7.2)	4.19 *m*	4.21 *m*			5.52 *dd* (7.1, 12.6)	5.47 *m*[a]	5.35 *m* (14.0)
9	6.40 *dd* (5.1, 16.1)	6.28 *dd* (6.1, 15.6)	6.15 *dd* (16.0, 6.0)	6.66 *d* (16.0)	6.85 *d* (15.4)	5.41 *dd* (6.9, 12.6)	5.47 *m*[a]	5.35 *m* (14.0)
10	5.78 *d* (16.1)	5.75 *d* (15.6)	5.90 *d* (16.0)	6.60 *d* (16.0)	6.54 *d* (15.4)	3.02 *d* (6.9)	3.12 br. *s*	2.02 *m*
11								1.34 *m*[a]
12								1.29 *m*[a]
13			5.63 *d* (10.8)		5.69 *d* (10.2)		5.47 *d*[a] (10.6)	1.29 *m*[a]

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Position	***1***	***2***	***3***	***4***	***5***	***6***	***8***	***9***
14	2.46 *s*		6.02 *dq* (10.8, 6.8)		6.10 *dq* (10.2, 6.6)	2.01 *s*	5.91 *dq* (10.6, 6.6)	1.30 *m*[a]
15		2.01 *s*	1.92 *d* (6.8)	2.07 s	1.95 *d* (6.6)		1.87 *d* (6.6)	0.90 *t* (7.0)

[a] Signals are overlapped.

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Table III. ^{13}C NMR spectral data [δ (ppm)] of compounds 1–9 (100 MHz, CDCl$_3$, TMS as reference). Source: Adapted with permission from references (*14*, *15*). Copyright 2006 Elsevier

Position	***1***	***2***	***3***	***4***	***5***	***6***	***8***	***9***
1	29.9	29.7	29.9	29.9	29.7	29.9	29.8	29.8
2	209.1	209.0	209.1	209.0	209.0	209.1	209.0	209.1
3	43.5	43.6	43.6	43.4	43.4	43.6	43.7	43.7
4	23.6	23.6	23.7	23.4	23.2	23.6	23.7	23.7
5	28.9	29.1	29.0	28.6	28.7	28.7	28.7	29.5
6	24.8	24.7	25.0	23.6	23.4	28.9	29.1	29.0[b]
7	36.6	36.8	36.8	40.9	40.7	27.0	26.9	27.0[c]
8	71.8	72.0	72.3	198.5	199.1	132.8	131.4	130.2[d]
9	122.0	148.5	144.8	122.1	123.3	122.0	124.5	129.4[d]
10	107.8	108.9	110.3	139.1	136.3	17.4	17.9	27.2[c]
11	71.9	72.3[a]	91.9	73.9	92.0	76.3	92.9	29.8[b]
12	74.1	75.1[a]	87.0	71.5	96.0	64.5	76.7	30.8
13	68.1	64.3	110.0	64.2	109.7	68.4	110.3	31.8
14	71.0	80.1	138.6	84.9	141.6	64.9	137.2	22.6
15		4.4	16.0	4.8	14.0		15.7	14.0

[a] [b,c,b,d] Assignments may be interchanged.

To unambiguously assess the stereochemistry of this secondary metabolite, the synthesis of the two isomerically pure forms (8*Z*,11*Z*)-pentadeca-8,11-dien-2-one (**10a**) and (8*Z*,11*E*)-pentadeca-8,11-dien-2-one (**10b**) was carried out (*17*). By comparison of the spectral NMR data of the natural compound *vs.* the synthesized isomers (Figure 6), it was observed that the olefinic resonances were not particularly indicative of the configuration. By contrast, allylic protons H-7, H-10 and H-13 were appreciably affected by the configuration of the double bond: in fact, moving from isomer **10a** (*Z* configuration) to **10b** (*E* configuration), protons H-10 (around δ 2.80 ppm) showed a 0.06 ppm upfield shift, while protons H-7 and H-13 in the region around δ 2.05 ppm, completely overlapped in the *Z* isomer, became significantly different in the *E* isomer, with a 0.08 ppm upfield shift of H-13 protons. The (8*Z*,11*Z*) configuration to pentadeca-8,11-dien-2-one (**10**) was finally assigned (*17*).

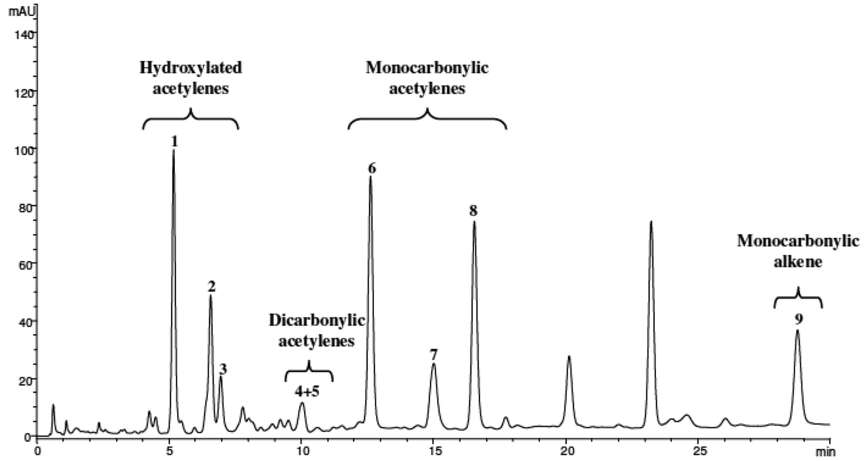

Figure 5. Chromatogram of a n-hexane extract of E. pallida roots on a LiChrospher RP-18 column (125 mm × 4.0 mm i.d., 5 µm). For peak identification see Figure 4. Adapted with permission from reference (17). Copyright 2008 The Royal Society of Chemistry.

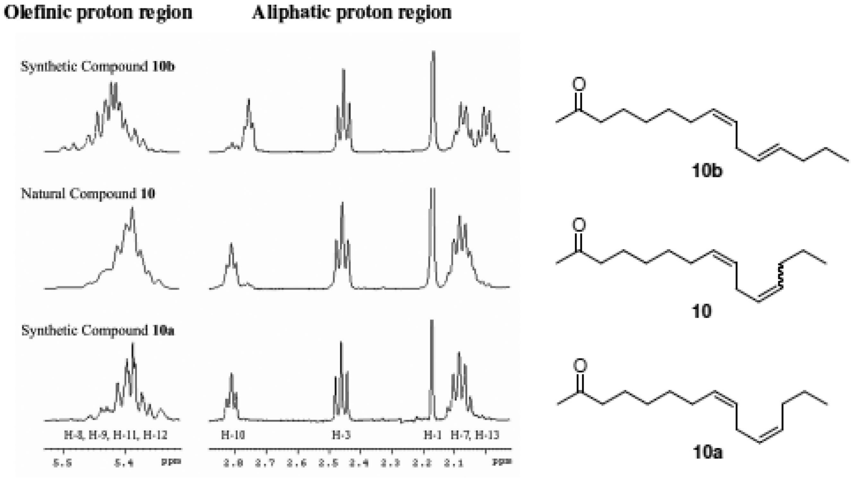

*Figure 6. Comparison of ^{1}H-NMR spectra of natural (**10**) and synthetic compounds (**10a,b**). Adapted with permission from reference (17). Copyright 2008 The Royal Society of Chemistry.*

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Secondary Assays

The secondary assays were carried out to determine the compounds responsible for the biological activity of *E. pallida* lipophilic extract. In particular, the secondary metabolites available in the purified form in higher amounts from *E. pallida* roots were tested for their cytotoxic activity on MIA PaCa-2 and COLO320 cancer cell lines in the concentration range 0.1-100 μM (*18*). Cell viability was evaluated by the WST-1 assay. The tested compounds caused a significant concentration-dependent decrease in both cancer cell viability after 72 h exposure (*18*). Table IV shows the IC_{50} values of the tested compounds, expressed as μM value (*18*).

Compound **8**, i.e. (8*Z*,13*Z*)-pentadeca-8,13-dien-11-yn-2-one, was found to be the most active on both the pancreatic and the colonic cancer cells. COLO320 cells appeared to be more sensitive than MIA PaCa-2 to all the isolated compounds, as previously described for the crude plant extract (*13*). In this cell line, the IC_{50} value of compound **8** (IC_{50} = 2.3 ± 0.3 μM) was significantly lower than that of the reference drug 5-fluorouracil (5-FU) (IC_{50} = 8.7 ± 0.2 μM) (*18*). The results of compound **8** seem to be relevant also on MIA PaCa-2 cells (IC_{50} = 32.2 ± 4.0 μM), due to the general low sensitivity of this cell type to therapeutic agents (*18*).

Table IV. IC_{50} values of the purified compounds from *E. pallida* roots on MIA PaCa-2 and COLO320 cells after 72 h exposure. Source: Adapted with permission from reference (*18*). Copyright 2008 John Wiley and Sons

Compounds	*IC_{50} (μM), mean ± S.E.*	
	MIA PaCa-2	*COLO-320*
2	>100	80.13 ± 2.21
3	>100	21.77 ± 1.22
4	60.91 ± 0.61	25.28 ± 0.55
5	63.53 ± 1.12	22.84 ± 2.12
8	32.17 ± 3.98	2.34 ± 0.33
5-Fluorouracil	7.42 ± 0.55	8.72 ± 0.18

Data are means from three independent experiments each run in triplicate.

In a further study (*19*), compound **8** revealed a concentration-dependent cytotoxicity on other human cancer cell lines, including leukemia (Jurkat and HL-60), breast carcinoma (MCF-7) and melanoma (MeWo). The IC_{50} values are shown in Table V. Compound **8** was found to be characterized by a good cytotoxic activity on leukemia Jurkat (IC_{50} = 2.1 ± 0.6 μM) and breast carcinoma MCF-7 (IC_{50} = 2.5 ± 0.7 μM) cancer cells. In MCF-7 cells, the IC_{50} value was lower than that of the positive control (5-FU). The greater sensitivity of Jurkat and MCF-7 cancer cells to the tested compound may be due to different drug-resistance properties of the cell lines used in this study (*19*).

Table V. IC_{50} values of compound 8 and positive control on human cancer cell lines after 72 h exposure. Source: Adapted with permission from reference (*19*). Copyright 2010 Georg Thieme Verlag KG

Compound	*IC_{50} (μM), mean ± S.E.M.*			
	Jurkat	*MCF-7*	*HL-60*	*MeWo*
8	2.1 ± 0.6	2.5 ± 0.7	21.3 ± 0.8	28.6 ± 2.3
Positive control[a]	0.9 ± 0.2	3.4 ± 0.5	0.8 ± 0.3	2.6 ± 0.9

Data are means from three experiments carried out in triplicate. [a] 5-Fluorouracil was the positive control for MCF-7 and HL-60; cisplatin was the positive control for Jurkat and MeWo.

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Furthermore, the tested compound showed selective effect on cancer cells *vs*. non-cancer cells, with an IC_{50} higher than 100 μM in human embryonic kidney HEK-293 cells (*18*).

Regarding the mechanisms of action of compound **8**, arrest of cell cycle in the G1 phase and induction of apoptosis were both found to be involved in the cytotoxic activity of this natural product (*18*, *19*). The capacity of compound **8** to arrest the cell cycle in the G1 phase was demonstrated on leukemia HL-60 cancer cells after 72 h exposure (*19*): at 20 μM (IC_{50}), 83% of the cell population was in the G1 phase; at 40 μM ($2\times IC_{50}$), 90% of cells were in the G1 phase.

The involvement of apoptosis in the mechanism of action of compound **8** was evaluated by the caspase 3/7 activity and the cytosolic internucleosomal DNA enrichment tests (*18*). As shown in Figure 7, after 24 h of incubation with the IC_{50} concentrations of compound **8** in MIA PaCa-2 (30 μM) and COLO320 (2.5 μM) cells, the caspase 3/7 activity increased 1.5-fold (*18*). Moreover, the cytosolic internucleosomal DNA fragments were significantly enhanced as well (2.9 and 2.3-fold higher than vehicle-treated cells) (*18*). Apoptotic cell death was thus found to be involved in the cytotoxic activity of the most active secondary metabolite. Extra-cellular DNA fragments were not detected in both cancer cell lines, thus indicating the absence of necrotic cell death as primary event involved in cytotoxicity induced by compound **8** (*18*).

Finally, the potential bioavailability of *E. pallida* lipophilic secondary metabolites was evaluated by using the Caco-2 monolayers (human colon carcinoma epithelial cell line), which is an accepted model of intestinal absorption. Of the acetylenes present in the *E. pallida* root extract, only three compounds were found to cross the Caco-2 monolayers, i.e. the hydroxilated acetylene **3** and two monocarbonylic acetylenes **6** and **8** (*18*). Therefore, the most active compound **8** has a good potential for intestinal absorption in humans after oral administration and it is likely to have an *in vivo* cytotoxic activity.

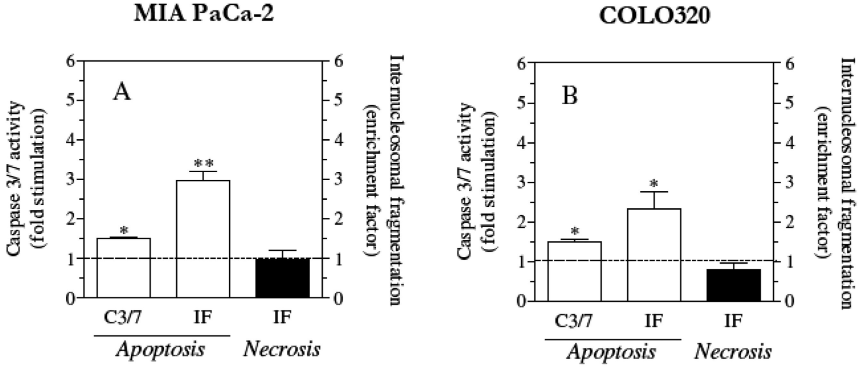

*Figure 7. Apoptotic and necrotic cell death after treatment with compound **8** for 24 h on MIA PaCa-2 (30 μM) and COLO 320 (2.5 μM) cancer cell lines. Reproduced with permission from reference (18). Copyright 2008 John Wiley and Sons.*

Structure-Activity Relationship

Considering the number of compounds isolated from *E. pallida* roots, a simple structure-activity relationship study based on the use of suitable molecular descriptors was carried out (*18*). While the simplest descriptors (such as molecular weight, molecular volume, ClogP) were not able to explain the trend of the biological activity data, more complex descriptors, such as the two contributions of the solvent-accessible surface area (SASA), i.e. SASA polar and non-polar components as well as combinations of them, showed a trend quite similar to the experimental activity data (*18*). The analysis of data obtained on MIA PaCa-2 cells indicated that the polar component of SASA follows a trend in accordance with the experimental IC_{50} values: lower values of polar SASA correspond to lower IC_{50} values (higher activity) (*18*). On the other hand, in COLO320 cells, a good correlation was given by the non-polar component of SASA and the biological activity: higher values of non-polar SASA correspond to lower IC_{50} values (higher activity) (*18*).

The analysis of the 3D molecular models of the tested compounds indicated only small conformational differences between the most active compound **8** and the other secondary metabolites isolated from *E. pallida* roots. However, the balance between polar and non-polar regions could be of relevance in explaining its greater activity: in fact, in the structure of this natural product there is a greater prevalence of non-polar areas. This property may influence its permeability through cell membranes and gives some advantages in interactions with the molecular target(s) (*18*).

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Total Synthesis

Due to the difficult purification of compound **8** from *E. pallida* roots, whose lipophilic extract contains many other constituents of similar polarity, and owing to the need of higher amount of this constituent for biological assays, its first total synthesis was recently undertaken (*20*). As shown in Figure 8, the retrosynthetic strategy was based on a Sonogashira coupling reaction for the insertion of the terminal alkene mojety. The enyne precursor **A** can be disconnected by chain extension and selective reduction to alkyne **B**, which can be traced back to commercially available 1-hexyne and propylenoxide. The desired product was synthesized in 11 steps in 25% overall yield (*20*).

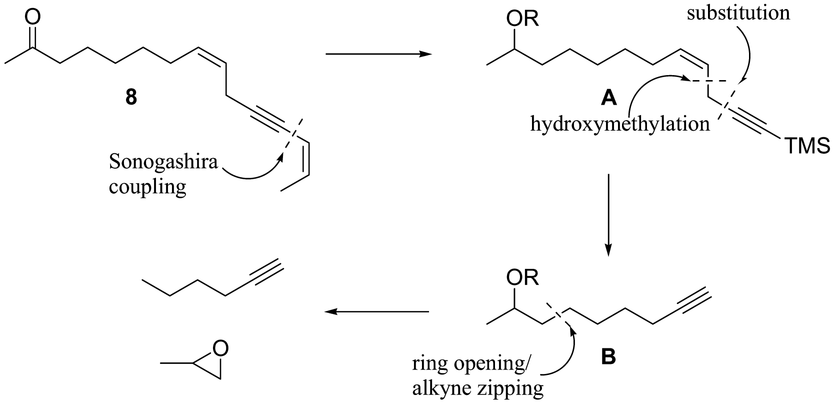

*Figure 8. Retrosynthesis of compound **8**. Reproduced with permission from reference (20). Copyright 2008 Elsevier.*

Phytochemical Analysis of *E. pallida* Plant Material and Natural Products

In another step of the research, a HPLC method was developed and validated for the first time for the phytochemical analysis of polyacetylenes and polyenes in *E. pallida* plant material and natural products (*15*). A reversed-phase monolithic stationary phase was selected in order to obtain a complete separation of the target analytes in a short time. The selected column has significantly greater porosity and hence permeability than conventional particulate stationary phases. The low back pressure inside the column allowed to work with an increased flow-rate, resulting in a faster separation. Figure 9 shows the HPLC chromatogram obtained by the analysis of a lipophilic extract from *E. pallida* roots (*15*).

Under the applied chromatographic conditions, the compounds of interest were separated in 20 min, with a reduction in the analysis time in comparison with conventional particulate stationary phases (Figure 5). The optimized method was

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validated in agreement with ICH guidelines and then applied to the quantitative analysis of the lipophilic secondary metabolites in *E. pallida* roots and dietary supplements (*15*). The results of the analysis of the plant material indicated that compound **1** was the main constituent of the hydroxylated acetylenes (0.31 mg/g). The dicarbonylic acetylenes (**4**,**5**) were present in low amounts in the roots. Compounds **6** and **8** were the most abundant monocarbonylic acetylenes (1.13 and 0.98 mg/g, respectively). The analysis of *E. pallida* dietary supplements on sale on the Italian market showed a great variability in the content of secondary metabolites. This highlights the importance of the developed method as a powerful tool for the phytochemical analysis and quality control of *E. pallida* plant material, extracts and commercial products.

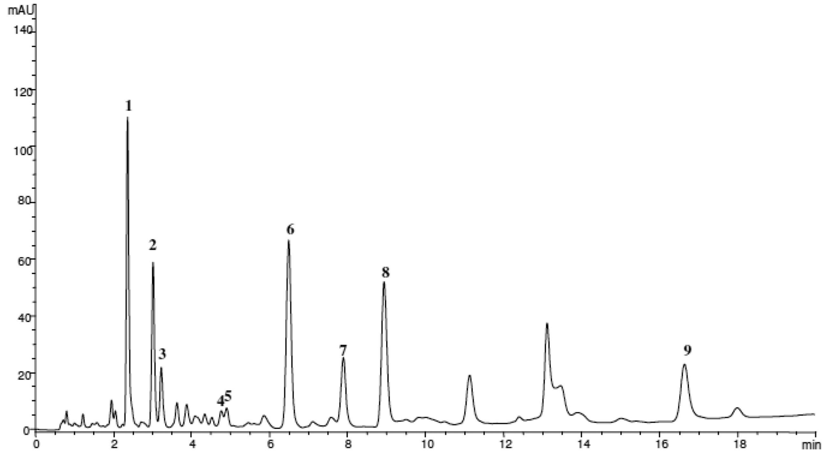

Figure 9. Chromatogram of a n-hexane extract of E. pallida roots on a Chromolith Performance RP-18e column (100 mm × 4.6 mm i.d.). For peak identification see Figure 4. Reproduced with permission from reference (15). Copyright 2006 Elsevier.

Stability Study

The application of the developed HPLC method to the analysis of *E. pallida* fresh roots did not reveal the presence of hydroxylated acetylenes **1**-**3** (*14*, *15*). However, compounds **1**-**3** were detected in high level when *E. pallida* ground plant material was stored for several days before the extraction (*14*, *15*). Therefore, compounds **1**-**3** can be considered as “artifacts”, due to an allylic oxidation reaction of the parent compounds **6**-**8** with molecular oxygen (*4*, *14*, *15*). The dicarbonylic compounds **4** and **5** might result from a further oxidation of the hydroxylated derivatives.

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Figure 10 shows the overall proposed scheme for the oxidation process of monocarbonylic acetylenes. The allylic oxidation reaction is quite slow in *E. pallida* crude extracts. On the other hand, the reaction is rapid for the purified compounds **6**-**8**, that should be stored under Ar at low temperature (-20/-80°C).

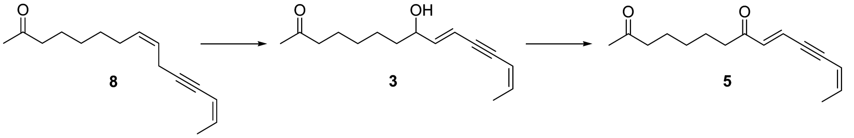

Figure 10. Proposed scheme for the oxidation of monocarbonylic acetylenes.

In order to assess whether the observed cytotoxic activity can be really attributed to compound **8** rather than to its oxidation products, its stability was studied by HPLC under the same conditions applied during the biological assays (*19*). In particular, a 100 mM DMSO stock solution of compound **8** was diluted with the culture medium at 100 µM concentration, incubated for 72 h at 37°C and sampled at fixed time intervals for HPLC analysis. The results indicated that the allylic oxidation of compound **8** proceeds via a hydroperoxide intermediate, which is then reduced to the corresponding alcohol. In this way, the double bond with *Z* configuration between carbons 8 and 9 is shifted in the position 9 with *E* configuration in the artifacts. Figure 11 shows the proposed mechanism for the allylic oxidation of monocarbonylic acetylenes.

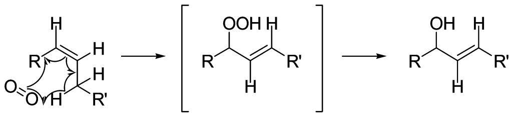

Figure 11. Proposed mechanism for the allylic oxidation of monocarbonylic acetylenes.

The HPLC stability study carried out to monitor the stability of compound **8** under the conditions applied in the biological assays indicated that its oxidation kinetic in the culture medium is quite slow: the percentage area of the hydroperoxide intermediate was about 25% after 72 h exposure (*19*). The final oxidation product, i.e. the hydroxylated derivative **3**, was not observed in the HPLC chromatograms after 72 h exposure (*19*). Considering that during the first 24 h of exposure the percentage area of the hydroperoxide intermediate is below 15%, the described cytotoxic activity can be mainly attributed to the genuine, not oxidized, compound **8** (*19*).

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Summary

In a search for cytotoxic compounds from plants of the genus *Echinacea*, *E. pallida* root extract showed higher cytotoxic activity in comparison with *E. purpurea* and *E. angustifolia*. This is in agreement with the different phytochemical profile of *E. pallida* if compared with *E. purpurea* and *E. angustifolia*. In fact, *E. purpurea* and *E. angustifolia* have alkylamides as the lipophilic constituents, whereas *E. pallida* contains lipophilic secondary metabolites with different structures. Through subsequent bioassay-guided fractionation, ten polyacetylenes and polyenes were isolated and characterized from *E. pallida* roots. Compound **8**, i.e. (8*Z*,13*Z*)-pentadeca-8,13-dien-11-yn-2-one, displayed good cytotoxicity and selectivity on human cancer cell lines and good potential for *in vivo* bioavailability. Arrest of cell cycle progression in the G1 phase and induction of apoptosis were demonstrated to be involved in the mechanism of action of this natural product. Due to the difficult purification of this compound from the plant material, its first total synthesis was also described. The stability of this molecule in the culture medium was monitored by HPLC, indicating that its cytotoxic activity can be mainly attributed to the genuine, not oxidized, molecule. Further studies are currently in progress to elucidate possible cellular targets (histone deacetylases, cyclin-dependent kinases etc…) involved in the cytotoxic activity of this natural product.

These findings indicate that polyacetylenes and polyenes from *E. pallida* are effective in inhibiting cancer cell proliferation. The incorporation of enriched fractions of these compounds in the diet may be useful in cancer prevention. The most active constituent also provides a possible lead structure for the development of new cancer therapeutic agents.

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Chapter 9

Wild Blueberries (*Vaccinium angustifolium*): Modulators of Vascular Function, Structure, and Metabolism

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We were the first to document, that dietary wild blueberries, high in phenolics and anthocyanins, affect the arterial biomechanical properties in Sprague-Dawley (SD) rats with functional endothelium and adult Spontaneously Hypertensive Rats (SHRs) with endothelial dysfunction. Wild blueberry diets decrease phenylephrine (Phe)-induced vasoconstriction through endothelium-related pathways, such as nitric oxide (NO) and may affect acetylcholine (Ach)-induced relaxation by modulation of the cyclooxygenase (COX) pathway and possibly the production/activity of COX-derived prostanoids in the adult SHR.

Furthermore wild blueberry-enriched diets result in structural alterations of aortic glycosaminoglycans (GAGs) such as concentration, redistribution and lower concentration of oversulfated disaccharides in galactosaminoglycan (GalAG) populations, affecting cellular signal transduction pathways and leading to a less atherogenic profile in the adult SHR. Thus, wild blueberry consumption may be important in regulation of vasomotor tone and atheroprotection with implications to preventing cardiovascular disease (CVD) and improving endothelial dysfunction.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Introduction

The protective role of polyphenolic compounds against cardiovascular disease (CVD) is documented by numerous animal, epidemiological and human intervention studies (*1*, *2*). Improvement of endothelial function and vascular tone by polyphenolic compounds, independently or in concert with their antioxidant properties, is one of the primary mechanisms involved in their vasoprotective effects (*1*). Evidence is also accumulating on the effect of polyphenols on the activity of matrix metalloproteinases responsible for extracellular matrix (ECM) remodeling (*3–6*) and on their role between ECM and vascular cell interactions important in the initiation and progress of atherosclerotic disease (*7–10*). Vascular tone is determined by the balance between endothelium-derived relaxing (EDRFs) and contracting factors (EDCFs) whereby under physiological conditions, EDRFs such as nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2), oppose the activity of EDCFs, such as endothelin-1 (ET-1), superoxide and cyclooxygenase (COX)-derived thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2), aiding in the maintenance of a functional endothelium (*11*). Alterations in the activity of NO and COX pathways leading to reduced vascular relaxations as well as increased release of vasoconstrictor prostanoids and reactive oxygen species contribute to endothelial dysfunction and vascular disease (*12*). The spontaneously hypertensive rat (SHR) is utilized as a model of endothelial dysfunction, a condition mostly observed in the adult and aged animal (*13*) and attributed to the augmented oxidative stress and reduced NO availability (*14–16*) as well as increased release and responsiveness of the vascular smooth muscle to COX-derived PGH_2 and TXA_2 (*17*). Additionally, adrenergic signaling associated with peripheral resistance is increased in the SHR (*18*, *19*). Endothelial dysfunction seems to be a common denominator of the development CVD (*12*). The ECM lining the endothelium, referred to as glycocalyx, is increasingly gaining appreciation for influencing vascular homeostasis and aiding in the maintenance of a functional endothelium (*20*). Glycosaminoglycans (GAGs) are prevalent components of the ECM that interact with numerous proteins and ligands to modulate crucial biological processes such as cell growth, development and signaling (*21*). Glycosaminoglycans are critical for vascular homoeostasis and function, explaining their emerging role as molecular targets for prevention of atherosclerosis (*22*). Hyaluronic acid (HA), heparan sulfate (HS), chondroitin (CS) and dermatan sulfate (DS), and to a less extent keratan sulfate (KS) are the main GAG molecules present in the vascular wall. Hyaluronic acid is not sulfated and not linked to a protein core, whereas HS and GalAGs occur as part of proteoglycan (PG) molecules that may contain more than one GAG type, such as HS and CS-containing syndecan, glypican or CS and DS-containing versican, decorin and biglycan (*23*). Hyaluronic acid and HS-PG confer protection on the vasculature (*24*), whereas CS/DSPGs are highly involved in the mechanisms associated with the development of cardiovascular disease and atherosclerosis in particular (*25*). The abnormal GAG profile documented in the SHR vascular wall, contributes to the dysfunctional vasculature of this animal model (*26–30*). Elevated peripheral resistance and blood pressure in this animal is related to the higher concentration of total GAGs and particularly CS, and a thicker subendothelial matrix in comparison with the normotensive Wistar Kyoto (WK) rat (*28*). Additionally, higher levels of sulfated PGs in resistance and conduit arteries occur before the development of the vascular dysfunction in the SHR (*30*, *31*). Wild blueberries, a rich source of anthocyanins and total phenolics (*32–34*), have been documented to confer beneficial properties for vascular function (*35–41*). Animal studies in our laboratory have demonstrated that wild blueberry consumption improves endothelial function and structure (*37–40*). In the normotensive Sprague Dawley (SD) rat, wild blueberry diets decreased vasoconstriction in response to the α_1-adrenergic agonist phenylephrine (Phe) with the involvement of the NO pathway (*37*, *38*) and altered the arterial GAG composition and structure towards a more vasculoprotective profile (*39*). In the young SHR, wild blueberry diets improved vasorelaxation in response to the muscarinic agonist acetylcholine (Ach) via modulation of the COX pathway (*40*). Therefore, we studied whether a wild blueberry-enriched diet can improve: a. the vascular tone in relation to phenylephrine (Phe)-mediated vasoconstriction (basal conditions) and Ach-mediated vasorelaxation (stimulated conditions), and b. arterial GAG concentration, distribution, composition and disaccharide profile in the adult SHR with established endothelial dysfunction.

Methods and Materials

The animal welfare and the experimental protocols were approved by the Animal Care and Use Committee of the University of Maine. Male SHRs (Charles River Laboratories, Wilmington, MA) at the age of twelve weeks were randomly assigned in one of two diets: control diet (C) (modified AIN-76), n = 10 and wild blueberry diet (WB) (control diet + 8% w/w freeze-dried wild blueberry powder substituting for dextrose), n = 10, for a period of eight weeks. The animals were housed in individual stainless-steel mesh-bottom cages in a room controlled for temperature (22°C) and light conditions (12:12-hour light: dark cycle) in the Small Animal Facility at the University of Maine. Tap water and food was provided *ad libitum*. Food consumption was measured daily and body weights weekly. The diets were prepared in our laboratory from purified diet ingredients as previously described (*40*, *41*). Wild blueberries, provided as a composite by Wyman's (Cherryfield, ME), were freeze-dried and powdered with standard procedures by FutureCeuticals (Momence, IL). The main anthocyanins, detected in the wild blueberry powder, malvidin 3-galactoside (Mv-3-gal) and peonidin-3-glucoside (Pn-3-glc), constituted 13% of the total anthocyanin (1.6 ± 0.2 mg/100mg) concentration (*32*).

Functional Arterial Property Studies

After eight weeks on the diets, rats were briefly anesthetized with 95% CO_2/5% O_2 and thoracic aortic rings were prepared and processed as previously described (*14*). Briefly, each aortic ring was suspended between two stainless-steel weightless wire triangles and placed in a 20 ml Radnoti tissue bath (Radnoti Glass Technology Inc. Monrovia, CA) filled with physiologic salt saline (PSS: NaCl

118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 12.5 and dextrose 11.1 mM) at 37°C and aerated with 95%O_2/ 5%CO_2 (pH 7.4). The isometric tension (g) developed in the aortic rings, was transmitted to a digital analyzer (Model 410 Micromed, Louisville, KY) and recorded in a personal computer by DMSI-210 software (Version 1.01 Micromed, Louisville, KY) (*40*, *41*). Rings were preconditioned for 10 min with Ach (10^{-8}M) and Phe (10^{-8}M) under baseline (preload, 1.5 g) and randomly assigned to treatment with either no inhibitor, or L-NMMA (10^{-4}M), NO synthase (NOS) I, II and III inhibitor, or mefenamic acid (10^{-5}M), COX I and II inhibitor. Vasoconstriction and vasorelaxation experiments were conducted with threefold concentration increments of the α_1-adrenergic agonist Phe (10^{-8}, $3x10^{-8}$, 10^{-7}, $3x10^{-7}$, 10^{-6}, $3x10^{-6}$, 10^{-5}M) and the muscarinic agonist, Ach (10^{-9}, $3x10^{-9}$, 10^{-8}, $3x10^{-8}$, 10^{-7}, $3x10^{-7}$, 10^{-6}, $3x10^{-6}$M) respectively, allowing a six-minute drug-tissue contact time at each agonist dose (*41*).

Arterial Glycosaminoglycan Studies

Aortic samples were defatted with methanol/chloroform solution (1:1, v/v) (*39*) and digested overnight at 37°C with PRONASE® (1.6 units/mg aortic tissue) in 50 mM Tris-HCl, pH 8.0.

They were then precipitated with ethanol and used to determine concentration of uronic acid (UA) by carbazole reaction as modified by Bitter and Muir (*42*) and total sulfated GAGs (sGAGs) with Blyscan™ colorimetric assay. The intra-differences in UA and total sGAGs within each diet group were not significant. Therefore all GAG samples obtained from each diet group were pooled, due to limited tissue availability from each rat and were applied to ion exchange chromatography on a DEAE-Sephacel column (7x1.6 cm i.d.). The column was eluted with a NaCl linear gradient (0.1 to 0.9 M, 10 volumes) and fractions of 0.8 ml were collected and analyzed for UA. The UA positive DEAE fractions containing distinct GAG populations were pooled, precipitated with ethanol and applied to cellulose acetate membrane electrophoresis with known standards of HA, HS, CS/DS to identify the GAG populations obtained by the DEAE fractions. Toluidine blue (TB) in 15% v/v aqueous methanol was applied for a 10 min membrane staining (*39*).

Glycosaminoglycan samples were further digested with a mixture of chondroitinases AC and ABC (0.01 units/10 μg UA) in 0.1 M sodium acetate – 0.1 Tris-HCl buffer, pH 7.3 and a mixture of heparin lyases I, II and III (0.05 units/25 μg UA) in 20mM acetate buffer, pH 7.0 with 1 μmol calcium acetate (*39*). After enzyme digestions, high performance capillary electrophoresis (HPCE) of GAG profiles was conducted on an $HP^{3D}CE$ instrument (Agilent Technologies, Waldronn, Germany) with a built-in diode array detector set at 232 nm, using uncoated fused-silica capillary tubes at 25°C. Samples were applied at the cathodic end under 50 mbar and 30 kV for 5 sec to achieve migration of Δ-disaccharides towards the anode by electrophoretic mobility and against the electroosmotic buffer flow (*39*, *43*).

Data and Statistical Analysis

The maximum force of vasoconstriction, Fmax (g) represents the highest value of each Phe concentration-response curve. The percent relaxation to the initial precontraction and the maximal vasorelaxation (%) was calculated from the highest response to each Ach concentration. Vessel sensitivity or reactivity, pD_2, was calculated as the negative $\log_{10}$ EC_{50}; EC_{50} was obtained by the semi-log transformation of the concentration response curves (*41*).

Two-way ANOVA was applied in ranked observations of food consumption, body weight, aortic tissue weight, Fmax, % vasorelaxation and pD_2 values, in the absence or presence of inhibitors, UA and sGAG concentration of aortic samples. Statistical analysis was performed with Sigmastat Statistical Program version 2.0 (SPSS Inc., Chicago, IL). Values are given as mean ± SEM (standard error of mean); differences are considered statistically significant at $p \leq 0.05$.

Results

Food intake and body weights were similar between the two diet groups (data not shown).

Functional Arterial Property Studies

The maximal vasoconstriction response (Fmax) to the adrenergic agonist Phe was significantly lower in the WB group in the absence of the inhibitors (Table I). Addition of L-NMMA (NOS I, II and III inhibitor) in the tissue bath resulted in no significant difference in the responses of the two groups but addition of MFA (COX I and II inhibitor), resulted in significantly lower Fmax in the WB group. In the absence of the above inhibitors, vessel sensitivity (pD_2) to Phe was similar in the two diet groups, but decreased in the presence of L-NMMA and increased in the presence of MFA in the SHRs that consumed wild blueberries (Table II).

A lower vasorelaxation in response to Ach was observed in the WB diet group compared to the C when no inhibitor was applied (Table III). Maximum vasorelaxation was similar between groups in the presence of L-NMMA, but incubation of vascular rings with MFA, resulted in a significantly greater vasorelaxation in the WB diet group (Table III). No significant differences in pD_2 values in response to Ach were detected between diet groups when no inhibitor was present, but after NO or COX pathway inhibition, significant increases in vessel sensitivity were observed in the WB diet group compared to the C group (Table IV).

Table I. Maximum vasoconstriction force[1], Fmax (g), in response to Phe in adult SHRs fed a control (C) or a wild blueberry diet (WB), (n = 10 rats per group) in the absence or the presence of either L-NMMA (10^{-4}M) or MFA (10^{-5}M)

Diet group	*Phe*	*Phe + L-NMMA*	*Phe + MFA*
SHR-C	1.00 ± 0.01	1.47 ± 0.04	0.79 ± 0.01
SHR-WB	0.83 ± 0.01*	1.42 ± 0.04	0.68 ± 0.01*

[1] Mean ± SEM; * Significantly different from the C group, $p \leq 0.05$.

Table II. Vessel sensitivity (pD_2)[1] in response to Phe in adult SHRs fed a control (C) or a wild blueberry diet (WB), (n = 10 rats per group) in the absence or the presence of either L-NMMA (10^{-4}M) or MFA (10^{-5}M)

Diet group	*Phe*	*Phe + L-NMMA*	*Phe + MFA*
SHR-C	7.65 ± 0.05	7.28 ± 0.02	7.41 ± 0.06
SHR-WB	7.78 ± 0.05	7.15 ± 0.02*	7.74 ± 0.06*

[1] Mean ± SEM; * Significantly different from the C group, $p \leq 0.05$.

Table III. Maximum % vasorelaxation[1] in response to Ach after initial Phe (10^{-6}M) precontraction in the adult SHRs fed a control (C) or a wild blueberry (WB) diet, (n = 10 rats per group) in the absence or the presence of either L-NMMA (10^{-4}M) or MFA (10^{-5}M)

Diet group	*Ach*	*Ach + L-NMMA*	*Ach + MFA*
SHR-C	94.63 ± 0.55	45.39 ± 0.50	97.76 ± 0.55
SHR-WB	91.93 ± 0.55*	46.41 ± 0.50	102.48 ± 0.55*

[1] Mean ± SEM; * Significantly different from the C group, $p \leq 0.05$.

Table IV. Vessel sensitivity (pD_2)[1] in response to Ach in adult SHRs fed a control (C) or a wild blueberry diet (WB), (n = 10 rats per group) in the absence or the presence of either L-NMMA (10^{-4}M) or MFA (10^{-5}M)

Diet group	*Ach*	*Ach + L-NMMA*	*Ach + MFA*
SHR-C	7.59 ± 0.02	7.04 ± 0.02	7.63 ± 0.02
SHR-WB	7.54 ± 0.02	7.17 ± 0.02*	7.72 ± 0.02*

[1] Mean ± SEM; * Significantly different from the C group, $p \leq 0.05$.

Arterial Glycosaminoglycan Studies

No significant differences were detected in UA concentration of the aortas isolated from either diet group, but the concentration of total sulfated GAGs (sGAGs) was significantly lower in the WB group, as compared to the C group (Table V). Three major GAG populations were identified in both diet groups, HS, GalAGs and HA. The total GAG concentration was similar between the diet groups, but the distribution of GAG populations was notably different. Heparan sulfate was 10% higher and GalAGs concentration was 11% lower in the WB aortas, while 25% increase in HA was observed in the WB compared to the C group (Figure 1). Disaccharide profile analysis of the isolated aortic GalAGs (Figure 2) revealed that Δ-di-mono4S, the main GalAG disaccharide in both diet groups, was 6% lower in the WB compared to C group. Also, Δ-di-mono6S, was 4% lower in the WB group, while the concentration of GalAG non-sulfated disaccharides (Δ-di-nonS) increased by 40%, and di-sulfated disaccharides (Δ-di-di(2,6)S) decreased by 58% in the WB group (Figure 2).

Table V. Dry aorta weight[1] and aortic uronic acid (UA)[2] and total sulfated GAG (sGAGs)[3] concentration in the adult SHR fed a control (C) or a wild blueberry (WB) diet

Diet group	*Aorta (mg)*	*UA (μg /mg)*	*sGAGs (μg /mg)*
SHR-C	10.4 ± 2.2	4.6 ± 0.3	13.7 ± 0.2
SHR-WB	10.0 ± 2.2	5.2 ± 0.3	12.6 ± 0.2*

[1] Mean ± SEM (n = 10); [2] Mean ± SEM (n = 6); [3] Mean ± SEM (n = 10); Values of UA and sGAGs represent concentration in dry defatted aortic tissue. * Significant difference form C group, $p \leq 0.05$.

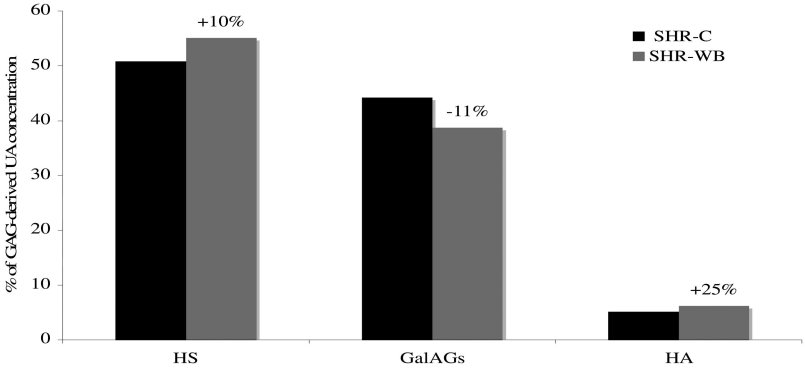

Figure 1. Percentage of GAG-derived uronic acid (UA) concentration¹ in each population isolated by anion-exchange chromatography on a DEAE-Sephacel column in the adult SHR fed a control (C) or a wild blueberry (WB) diet (n = 10) ¹µg UA/mg of dry defatted aortic tissue HA: hyaluronic acid, HS: heparan sulfate, GalAGs: galactosaminoglycans.

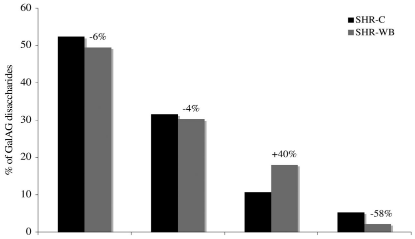

Figure 2. Percentage of GalAG Δ-disaccharide composition, determined by HPCE analysis following combined digestion with chondroitinases AC & ABC of aortic samples from adult SHRs fed a control (C) or a wild blueberry (WB) diet, (n = 10 rats per group).

Discussion

Functional Arterial Property Studies

Agonist induced vasoconstriction and vasorelaxation was examined in adult, 20-week old SHRs after eight weeks of a control or a wild blueberry-enriched diet. We observed that wild blueberries modulate the NO pathway under basal conditions (Phe-induced vasoconstriction) and potentially the COX pathway under stimulated conditions (Ach-mediated vasorelaxation).

A significant reduction in aortic tension was documented in the adult SHR with established endothelial dysfunction when challenged with the adrenergic agonist Phe. The diminished Phe-induced vasoconstriction in SHRs that consumed wild blueberries was abolished when L-NMMA was present, indicating involvement of the NO pathway under basal conditions.

Additionally, lower vessel sensitivity to Phe was observed in the wild blueberry diet group compared to the control in the presence of the NOS inhibitor. The COX pathway does not seem to be a major participant in Phe-induced vasoconstriction, based on the observation that MFA application affected SHR aortic tension similarly in both diet groups, although a significantly higher vessel sensitivity to the adrenergic agonist was observed in the wild blueberry group in the presence of MFA.

The maximum response to Ach was lower in the WB group; but the inhibition of the NO pathway resulted in a similar response in both diet groups, even though vessel sensitivity to Ach was increased. Inhbition of the COX pathway, resulted in a greater relaxation in response to Ach, which was accompanied by higher vessel sensitivity to the muscarinic agonist. This indicates that the effect of wild blueberries on Ach-induced vasorelaxation may be mediated by the COX pathway in the adult SHR, but the excessive release of COX derived vasoconstrictors in this model, at this age, most likely masks the effect of wild blueberries when the aorta is challenged with the muscarinic agonist Ach. Studies suggest that in the SHR, NO bioavailability is compromised due to increased superoxide release, despite an enhanced NOS expression, leading to endothelial dysfunction (*14–16*). Overall, the balance between vasorelaxant and vasoconstrictor factors in the SHR is shifted towards higher vasoconstriction (*11*), which is exacerbated by age (*12*, *13*).

Our wild blueberry diet improved NO-mediated vasorelaxation in response to Phe or low Ach concentrations while it enhanced Ach-induced vasorelaxation only when the COX pathway and prostanoid release was inhibited. These observations may be explained by the reduced NO bioavailability, inadequate antioxidant defense (*14–16*) as well as increased COX-mediated vasoconstriction (*17*) in the SHR.

Thus, the reduced vasoconstriction response to the adrenergic stimulus elicited by the wild blueberry diet is an especially important finding in light of the increased adrenergic signaling and peripheral resistance in the SHR (*18*, *19*). Studies utilizing aortic ring incubation with single bioactive compounds have documented similar results with our wild blueberry diet studies i.e. reduced Phe-induced aortic tension, but the above compounds were added in the tissue baths in pharmacological doses (*44–46*).

Additionally, studies in SHRs that document an increase of Ach-induced vasorelaxant response elicited by bioactive compounds, such as quercetin and chlorogenic acid, have been administered or applied directly in the tissue bath in pharmacological concentrations (*47–49*). One should be cognizant that metabolism of a bioactive compound can alter its vasoactive properties (*49*, *50*) and polyphenols are known to be converted into various metabolites after ingestion (*51*). In our dietary studies, we demonstrate that wild blueberries consumed for eight weeks, modulate the NO pathway under basal conditions (Phe-induced vasoconstriction) and potentially the COX pathway under stimulated conditions (Ach-mediated vasorelaxation) by modifying agonist-receptor interactions and the contractile response of the vascular smooth muscle in the SHR.

Arterial Glycosaminoglycan Studies

We document for the first time that a wild blueberry-enriched diet can alter aortic GAG composition and structure in the SHR model of endothelial dysfunction. We observed a significant reduction of total sulfated aortic GAGs in the adult SHR after eight weeks of the wild blueberry diet. Additionally, wild blueberry consumption resulted in a redistribution of GAG populations by increasing HA and HS concentrations, by 25% and 10% respectively, and decreasing GalAGs by 11%. The disulfated and monosulfated GalAG disaccharides were reduced as a result of the wild blueberry diet, whereas a higher percentage of non-sulfated GalAG disaccharides was detected in the SHR aortic tissue.

Hyaluronic acid is crucial for the structural integrity of the arterial glycocalyx (*20*). Increased concentration of aortic HA due to diuretic treatment has been positively associated with total systemic compliance (*52*). Additionally, HA is involved in flow-induced endothelial NO, by sensing and transferring the shear stress to the endothelium (*53*). Similarly to HA, HS mediates NO synthesis and release in response to shear stress on the luminal surface of the vessel wall (*54*) and furthermore, PGs containing HS, such as perlecan, prevent oxidized LDL and monocytes from binding to ECM and inhibit smooth muscle cell proliferation (*55*). The increased HA and HS concentrations that resulted from the wild blueberry consumption in our studies, is in agreement with our observations from the functional arterial studies in the adult SHR that document a NO pathway-mediated attenuation of the vascular tone in response to an adrenergic stimulus. In the SHR, the elevated peripheral resistance and blood pressure is related to higher vascular PG and CS synthesis, leading to the hypertrophy of the aortic medial layer (*28*, *56*). Hypertrophy of the aortic media observed in young SHRs before the development of hypertension is most likely responsible for the lower aortic wall compliance (*57*). In addition to their role in the development of hypertension, GalAGs promote atherogenesis, with CS and DS proteoglycans such as biglycan, highly involved in LDL binding to arterial PGs (*58*, *59*), subendothelial retention and atherogenicity (*25*, *60*). Here, we document a lower GalAGs concentration after eight weeks of wild blueberry consumption, hence a vasculoprotective role of the wild blueberry diet.

Besides the beneficial redistribution of GAG populations, the wild blueberry diet attenuated sulfation of the SHR aortic GAGs. Modification of PG synthesis by sulfate incorporation occurs before the establishment of vascular dysfunction and hypertension in the SHR (*30*). Higher sulfate incorporation into aortic PGs is observed in the SHR versus the WK rat (*29*), and in other models of experimental hypertension such as the Dahl rat (*61*). The binding affinity of LDL particles to PGs also depends on the degree of sulfation (*59*). Hence, statin-induced, lower LDL retention into smooth muscle cells is explained by a lower degree of PG sulfation (*62*).

The population redistribution and decrease in sulfated GAGs in the SHR aorta after eight weeks of wild blueberry consumption, suggests that wild blueberries play an important role in aortic GAG remodeling and may improve structural characteristics of the vascular wall related to the development of endothelial dysfunction eventually leading to atherosclerosis. This is an important finding, in light of the emerging role of GAGs as vascular targets for prevention of CVD and atherosclerosis with special attention given to their chain length and sulfation pattern (*22*).

Wild blueberries are a rich source of bioactive polyphenols and anthocyanins, but also a good source of manganese (*63*), a trace element documented to decrease sulfation of HS in the SD aorta (*64*). Consumption of wild blueberries may alter GAG synthesis and turnover through an effect on enzymatic processes and related signaling pathways, as suggested by studies on tea polyphenols (*9*), plant-derived micronutrients (*10*), flavonoids (*65*), sorghum phenolics (*66*) and genistein (*67*), reported to positively modulate GAG metabolism with effects on vascular remodeling and maintenance of aortic function.

Past dietary animal studies in our laboratory have documented the capacity of wild blueberries to improve endothelial function and GAG profile in the SD rat with functional endothelium (*37–40*). Therefore, wild blueberry incorporation in the diet can benefit vascular health in a preventive or therapeutic manner depending on the state of the endothelium. We were the first to report the role of wild blueberry diets on improving the vasomotor tone and structural components of the aortic vascular wall in the SHR. The reduced vasoconstriction and the GAG population redistribution and decreased sulfation in the adult SHR with established endothelial dysfunction reflect the potential of wild blueberries to modulate critical components of vascular function and structure with implications for prevention and/or treatment of vascular disease.

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Chapter 10

Carrot Bisacetylenic Oxylipins – Phytochemicals Behind the Mask of the Superfood

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Carrots are an important vegetable of the American diet and contain numerous bioactive phytoalexins. Polyacetylenes are bitter tasting bisacetylenic oxylipins in the family of plants known as Umbelliferae or Apiaceae. Carrots contain three predominate polyacetylenes which include falcarinol, falcarindiol, and falcarindiol 3-acetate. The concentration of polyacetylenes in colored carrots varies by as much as seven fold and may be dependent upon growing conditions, root tissue anatomy, year harvested, and storage and processing conditions. Polyacetylenes have traditionally been extracted with ethyl acetate or dichloromethane, or more recently by supercritical fluid extraction. Carrot polyacetylenes possess allelopathic activity which may explain the historical health benefits of carrots since studies investigating β-carotene doesn't seem to adequately explain the reduced risk of certain types of cancer. Polyacetylenes are cytotoxic to certain fungi, microorganisms, and protective against various cancer cells in both *in vitro* and *in vivo* studies. Polyacetylenes possess bioactive properties which include the inhibition of lipid transport enzymes, induction of liver phase II detoxification enzymes, and anti-inflammatory activity. Carrots should be included in the top superfoods list due to their high dietary prevalence, wide variety of potent bioactive secondary metabolites, and increasing level of scientific support for human health.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

The Identity of a Superfood

The term "superfood" is widely used to highlight different classes of food as having superior effects on human health over other non-superfoods. There are several proposed definitions for the term:

- Foods that confer health benefits due to high concentrations of phytochemicals.
- Foods that provide multiple disease-fighting nutrients and are easy to include in everyday meals.
- Foods that have a high nutrient, vitamin, and mineral content and may be used as a replacement for supplements by adding them to a diet.
- Superfoods may help people feel more energetic, provide protection against disease, and promote a healthy lifestyle.

Examples of commonly consumed superfoods can be found across several different food groups including beverages (tea, red wine), fruits (avocadoes, blueberries, pomegranates, cranberries, oranges, tomatoes), vegetables (broccoli, spinach, onion, beets, pumpkin), seeds/nuts (almonds, walnuts, flax), other (soy, omega-3 fish, olive oil, whole grain, cinnamon, dark chocolate, garlic, yoghurt/probiotics, beans).

Several of the above listed foods are associated with good scientific support for their impact on human health. For example, polyphenols in green tea (*1*) and cranberries (*2*) show good support for the prevention of cardiovascular disease. Intakes of dietary components such as olive oil, whole grains, and fish high in omega-3 fatty acids are generally associated with healthier lifestyles and consequent reduction in disease risk (*3*, *4*). Other listed superfoods such as pumpkin and beets are associated with a smaller body of scientific evidence and less frequent consumption.

The health benefits of superfoods are as diverse as the list of identified foods. There is growing scientific support and understanding of phytochemical components in superfoods and their potential to prevent or reduce inflammation, regulate metabolism, lower blood cholesterol and serum lipids, protect against heart disease and cancer, up regulate detoxification enzymes, and promote digestive health.

Carrots in the Modern Diet

Carrots are most often missing from the list of common superfoods. This is despite the fact that in 2006 it was determined that carrot consumption was equivalent to 12 pounds/person/year and is an increasing way to meet the dietary recommendation for increased vegetable consumption. Carrots are often offered as an alternative healthy snack or school lunchbox staple. Today carrots may be found in many forms which include fresh market, baby, canned, frozen, dehydrated, and juiced. There is an increasing public awareness of different commercial varieties of carrots. Supermarkets and farmers markets have begun to offer colored carrot varieties that are very diverse and rich in phytochemical composition.

Carrots have been characterized to contain more than 47 anti-inflammatory chemicals, 51 different antioxidants, 23 hypocholesterolemic compounds, and 52 antibacterial compounds (*5*). The health benefits of carrots have most often been researched in context of the carotene or pre-vitamin A content. A review of literature supporting the health benefits of carrots yields supportive data looking primarily at carotene content as the hypothesis for mechanism of action.

The majority of studies looking at carrots as an intervention in animal and human studies is most often centered on a hypothesis of carotenoid content as a mechanism of action. While there is little doubt that carotenoids have impact on human health, there is little to no recognition of other phytochemical constituents in carrots that may enhance or negate studied biological activity. For example, colored carrots containing carotenoids increase liver vitamin A stores and antioxidant capacity compared to white carrots devoid of carotenoids (*6*). Carotenoid intake from fruits and vegetables, of which carrots are the predominant source, in 1300 elderly subjects was associated with decreased cardiovascular risk (*7*). Serum concentrations of carotenoids were found to be positively associated with adiponectin concentrations in 437 subjects, an association thought to reduce cardiovascular disease (CVD) risk (*8*). An inverse relationship was found between serum carotenoids and endothelial dysfunction and inflammation in human subjects (*9*). While it is true that carrots contain significant levels of carotenes, and carrots have a positive impact on human health; what is often not recognized is the large diversity of other phytochemical constituents in carrots and their potential role in human health.

The health benefits of carrots have largely been driven by epidemiological data looking at risk vs. serum concentration of carotenoids. Due to the high prevalence of carrots in the diet and consequent large contributing carotenoid pool and ease of measure, carotenoids are often targeted as the bioactive phytochemicals in carrot. A linear thought process in understanding the health benefits of carrots often proceeds as outlined by Young et al. (*10*):

- A diet high in fruits and vegetables is associated with a decreased risk of certain cancers.
- Epidemiologic data supports a correlation between serum β-carotene and decreased cancer risk.
- In European and North American diets, carrots account for more than half of β-carotene intake.
- Therefore β-carotene is likely to be the bioactive phytochemical in carrots responsible for reduced cancer risk.

Despite the suggestive hypothesis described above, β-carotene supplementation has not shown protection against the disease (*11*, *12*). These negative findings may alternatively suggest a mechanism of action by other phytochemical classes or mixtures of phytochemicals that may work additively or synergistically on human health. There is increasing interest in other less prevalent secondary metabolites in carrots and their effects on human health.

Carrot Allelochemicals

Carrots and other vegetables in the Apiaceae family contain numerous potent bioactive compounds termed allelochemicals. Allelochemicals display characteristic properties of allelopathy, which is the production of secondary plant defense compounds which have no effect on the plants growth or reproduction but negatively impact foraging of the plant. These natural pesticides may ward off attack by fungi, bacteria, nematodes, and mammals. Secondary compounds are typically bitter in taste, and as in carrots, located in the outer most region of the peal. In very high concentrations, allelochemicals may cause skin irritation or be neurotoxic. Examples of these types of phytochemical classes range from alkaloids, tannins, saponins, and some polyphenols. Some compounds such as glucosinolates, which form the highly pungent isothiocyanates, show selective cytotoxicity against cancer cells and are supported by strong scientific data (*13–15*). “Phytoalexin” is a similar related term used to describe phytochemicals or antibiotics produced by plants in response to injury or environmental stress (*16*). These allelochemicals may affect human physiology and disease even though they are not considered “traditional essential nutrients”. Characteristically secondary plant defense compounds can be highly potent in their effect on biological systems.

A second characteristic often exhibited by secondary plant metabolites is a dose response phenomenon known as hormesis. Hormesis is a differential response to a toxin dependent on dose. This is classically illustrated by looking at relationships between alcohol intake and all-cause mortality and related health challenges (*17*). Light or moderate drinkers have lower mortality and other health burdens compared to heavy drinkers or abstainers. Moderate alcohol consumption has been shown to have health benefits, compared to higher doses of alcohol which acts as a toxin. Additionally, there is a growing field of research studying low doses of radiation and its beneficial aspects on biological systems (*18*, *19*). As a third example, consider that glucosinolates in high concentrations are known goitrogenic compounds but in low dose concentrations are supported by arguably the largest body of scientific evidence within natural product research supporting anticarcinogenic properties.

Carrots contain a number of secondary plant defense compounds such as the bisacetylenic oxylipins, 6-methoxymellein (6-MM), eugenin, gazarin, and the phenylpropanoids. 6-MM has been identified as one among many bitter component in carrots (*20*, *21*) induced by environmental stressors such as UV-irradiation or pathogen inoculation (*22*). 6-MM is a known strong antifungal agent (*23*). Eugenin has been shown to be cytotoxic in tumor cell lines (*24*) as well as a group of compounds known as phenylpropanoids (*25*).

Bisacetylenic oxylipins are predominate bitter compounds in carrots (*26*). Bisacetylenic oxylipins, otherwise known as polyacetylenes, contain two acetylenic bonds in a C17 chain with hydroxylated end groups. These compounds, which are the focus of this chapter, are derived from the synthesis pathway of unsaturated fatty acids. There are more than 1400 different polyacetylenes and related compounds isolated from higher plants, primarily from the Apiaceae family of plants. Carrots contain three major polyacetylenes; falcarinol, falcarindiol, and falcarindiol 3-acetate, see Figure 1. Recently, nine other polyacetylenes were identified in carrot in less abundance than those in Figure 1.

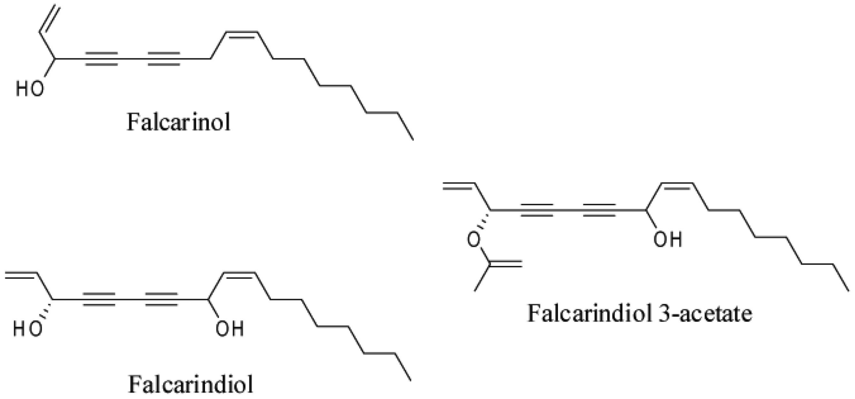

Figure 1. Structures of the three main bisacetylenic oxylipins or polyacetylenes in carrot.

Extraction, Isolation, and Analysis of Carrot Polyacetylenes

There are a number of published methods for the solid/liquid extraction of carrot polyacetylenes. The most common methodology is solvent extraction with dichloromethane, ethyl acetate, diethyl ether, or methanol. Extraction can be done in either fresh or lyophilized material with stirring, ultrasonication, or pressurized liquid extraction (*27*) and more recently ultrasonic liquid processing (*28*). Recently in our lab, we performed supercritical fluid extraction (SFE) of carrot polyacetylenes with good success. After extraction at 621 bar, with a run time equivalent to 30g CO_2 per g dried carrot and ethanol co-solvent, the remaining concentration of polyacetylenes in the extracted (defatted) carrot was 0.1, 0.4, 0.1, and 0.4% of the concentration in the non-extracted purple carrot raw material for falcarindiol, falcarindiol 3-acetate, falcarinol, and vitamin E respectively (unpublished data). This indicates that SFE is a potential industrial scale method for extraction of polyacetylene compounds from carrots.

Previously described methods of analysis for polyacetylenes include characterization by the unique UV-spectral properties (*29*, *30*), nuclear magnetic resonance (NMR) spectroscopy analysis (*31*, *32*), and *in situ* analysis by raman spectroscopy to localize the anatomy of polyacetylenes in carrot cross-sections (*33*, *34*). A number of different mass spectrometry methods have been successfully applied which include GC-MS (*35*) with chemical (CI) and electron ionization (EI) (*36*, *37*), fast atom bombardment (FAB-MS) (*38*), and electrospray ionization (ESI-MS) in both positive and negative mode ionization (*28*, *32*, *39*).

Polyacetylene Concentrations Are Variable by Carrot Cultivar

Carrots are the predominate source of polyacetylenes in the human diet. Other less prevalent food sources include celery, parsley, parsnips, fennel, and celeriac. Carrot concentration differences are dependent upon a number of factors including cultivar (*37*, *40*), physiological age and geographical location (*40*), climate (*41*), and storage and processing conditions (*42*). While environmental conditions may alter the polyacetylene content, carrot variety is the largest contributor to variability. The concentration of total polyacetylenes in different colored carrots was shown to vary by more than four fold in a study of sixteen different varieties (*37*), while others found almost a 6 fold difference in 27 different carrot genotypes (*27*).

As an example of varietal differences, Table I indicates the concentration of falcarindiol can vary by as much as 7 fold in different colored carrots. Other notable differences include the high level of falcarinol and total polyacetylenes in the Deep Purple cultivar compared to 13 other colored carrot varieties. Commercial baby carrots contain the least total concentration of polyacetylenes. Wild carrot, or Queen Anne's lace, has the second highest content of polyacetylenes, while white carrot varieties (White Satin, Crème de Lite) are a richer source of the polyacetylene falcarindiol 3-acetate.

In addition to the diversity of polyacetylene concentrations in colored carrots, they also contain variable amounts of other phytochemicals including carotenoids, total phenolics, anthocyanins, alpha-tocopherol, terpenes, sesquiterpenes, and other antioxidants (*37*). Commercial market carrots contain higher amounts of carotenes, while other phytochemicals like phenolics are much higher in purple carrot varieties. It is likely that there is large variability in other bioactive secondary plant compounds, dependent on carrot variety, commonly known to increase due to environmental stress.

Stability of Carrot Polyacetylenes

The stability of carrot polyacetylenes is dependent upon storage conditions, since long term storage of fresh and steam-blanched carrots at 1°C leads to reduced falcarinol levels. Boiling leads to significant reductions in concentration, while storage at -24°C preserves the polyacetylene content. The extracted polyacetylenes are sensitive to oxidation or enzymatic degradation, heat, and light exposure (*42*). However, others have found increases in polyacetylenes and stable amounts of the secondary metabolite 6-methoxymellein in fresh carrots stored at 1°C for four months (*40*). The use of Raman spectroscopy mapping of carrot slices during storage allows for a quick understanding of the changes that occur to polyacetylenes and other constituents such as starch, pectin, cellulose, and lignan during storage (*43*). Recently Rawson and others determined that freeze drying carrots disks better preserved both carotenoids and polyacetylenes when compared to hot air drying. Pre-treatment of carrots with ultrasound enhances retention of the polyacetylenes compared to blanching of carrots prior to drying. The enhanced retention of polyacetylenes by ultrasound may be related to increased extraction of the compounds from the matrix during analysis (*44*).

Table I. Polyacetylene diversity in cultivated colored and commercially available carrots. (Adapted with permission from reference (*49*). Copyright 2009 ACS Publications.)

	Concentration (mg/g, dry weight basis)			
Carrot Varietal	*FaDOH*	*FaDOH 3-Ac*	*FaOH*	*Sum*
Commercial baby carrots	142 ± 6	308 ± 36	236 ± 3	686 ± 43
Commercial market carrots	368 ± 10	1090 ± 17	602 ± 2	2060 ± 27
Atomic Red	101 ± 7	261 ± 8	331 ± 4	693 ± 3
Snow White	82 ± 2	359 ± 15	291 ± 8	731 ± 14
Amarillo	112 ± 10	389 ± 11	257 ± 15	759 ± 35
Purple Haze	129 ± 14	339 ± 34	438 ± 68	906 ± 115
Dragon	307 ± 42	367 ± 31	369 ± 50	1043 ± 123
White Satin	182 ± 34	721 ± 40	245 ±20	1148 ± 83
Crème de Lite	190 ± 35	678 ± 63	391 ± 41	1259 ± 106
St Valery	228 ± 10	484 ± 21	566 ± 20	1277 ± 51
Lunar White	418 ± 24	411 ± 17	543 ± 22	1371 ± 63
Danvers	251 ± 5	605 ± 2	552 ± 9	1409 ± 15
Cosmic Purple	518 ± 7	560 ± 14	386 ± 2	1465 ± 20
Yellowstone	270 ± 26	704 ± 20	609 ± 23	1583 ± 66
Queen Anne's Lace	583 ± 26	737 ± 24	399 ± 13	1719 ± 63
Deep Purple	491 ± 10	970 ± 21	1553 ± 29	3015 ± 56

Other external factors such as harvest date, storage, planting location, cultivar, and year harvested produced different degrees of variation in polyacetylene content in Swedish carrots. Falcarindiol and falcarindiol 3-acetate levels were higher in carrots harvested early in the growing season, while falcarinol did not change. Fresh carrots had higher levels of polyacetylenes compared to stored carrots (1°C) harvested at the same time period. Polyacetylene levels increase or decrease in cold storage dependent upon the starting fresh concentration and cultivar. Growing location has an effect on concentration, but year to year variance was shown to have the largest influence on polyacetylene content (*45*).

Biological Activity of Polyacetylenes

The polyacetylenes, like other bitter carrot secondary compounds mentioned above, possess potent antifungal and antibacterial properties (*46*)(*47*). Polyacetylenes inhibit and activate a number of different enzymes involved in serum lipid metabolism such as diacylglycerol acyltransferase (*48*), cholesterol ester transfer protein (*49*) and acyl-CoA:cholesterol acyltransferase (*50*). The polyacetylenes also inhibit enzymes involved in inflammatory signaling such as inducible nitric oxide synthase (iNOS) (*51*), lipoxygenase (*52*), cyclooxygenase-I (*53*), 15-hydroxyprostaglandin dehydrogenase (*54*), and cytokines IL-6 and TNF-α (*55*).

Other noted biological activity includes anti-platelet aggregation effects due to inhibition of thromboxane formation (*56*), and inhibition of the development of pre-neoplastic colonic lesions in a rat model (*57*).

Polyacetylenes up-regulate several liver detoxification genes (*58*) and quinone reductase activity (*59*). As an example of hormesis, falcarinol stimulates primary mammalian epithelial cells at concentrations between 0-0.04 μM and is toxic at concentrations greater than 4 μM (*42*). Falcarinol is the most active polyacetylene with reported cytotoxic effects against an acute lymphoblastic leukemia cell line with an IC_{50} value of 3.5 μmol/L (*60*). As another example of the polyacetylenes hormetic potential, low concentrations (1.6-25 μM) of polyacetylenes increase cytosolic glutathione peroxidase (cGPX) transcription and decrease heat shock protein (HSP70) and heme oxygenase 1 (HO1) in a primary myotube model of H_2O_2 induced stress. At concentrations of 50 and 100 μM, cGPX transcription is reduced while HSP70 and HO1 transcription increases indicating an adverse treatment effect of the polyacetylenes at higher concentration (*61*).

There is a growing body of evidence that supports the anti-carcinogenic properties of polyacetylenes due to selective cytotoxicity against various cancer cells (*60*, *62–64*). The selective cytotoxic effects have been measured to be 20x's more potent compared to non-cancerous cells (*65*). The antitumor activity was investigated in several human cancer cell lines, such as ovarian (SK-OV-3), skin melanoma (SK-MEL-2), and colon (HCT-15) cells (*66*). The cytotoxicity of polyacetylenes was investigated through induction of apoptosis in human colon, pancreatic, epidermoid, larynx, and lung carcinomas (*67*).

Recently, carrot juice extract from juiced carrots was investigated in 4 myeloid, 4 lymphoid leukemia, and 1 non-tumor hematopoietic stem cell line. The study hypothesis suggested that carrot juice as an alternative treatment is effective at killing or stopping proliferation of cancer cells due to the complexity of bioactive components, mainly β-carotene and polyacetylenes. β-carotene has previously been shown to induce cell cycle arrest in G0/G1 phase after 12-hour treatment of a HL-60 myeloid leukemia line (*68*), while the polyacetylenes as indicated in the paragraph above indicate strong activity in a number of human cancer cell lines. The carrot juice extract induced apoptosis in all myeloid and lymphoid human leukemia cell lines after 24, 48, and 72 hours and matches previous findings on polyacetylenes. There was a clear difference between the leukemic cell lines and non-tumor cells. The lymphoid leukemia cell lines appeared more sensitive to carrot juice extract than myeloid cells (*69*). This recent paper highlights the importance of the carrot as food and its importance in supporting human health, specifically anti-carcinogenic potential, in a minimally processed form such as juice.

Carrots contain a diversity of anti-inflammatory compounds which may also function as allelochemicals. Research in our lab has focused on characterizing the anti-inflammatory activity of carrots attributable to the polyacetylenes. The polyacetylenes vary vastly by cultivar and our research has focused largely on the Deep Purple carrot variety due to its higher concentrations of polyacetylenes.

The bioactivity of colored carrot varieties is largely correlated with the polyacetylene content, but other secondary metabolites or phytochemicals in carrots are also likely to contribute to anti-inflammatory activity. This is illustrated by the fact that Queen Anne's lace, commercial market carrots, commercial baby carrots, and Deep Purple carrot are the most potent inhibitors of lipopolysaccharide (LPS) induced nitric oxide in macrophage cells as indicted by their IC25 values, Table II. This is despite the fact that Queen Anne's lace and Deep Purple contain high concentrations of polyacetylenes, and commercial market and baby carrots contain lesser amounts as seen in Table I.

Table II. Inhibition of nitric oxide production in macrophage cells (IC25). (Adapted with permission from reference (*49*). Copyright 2009 ACS Publications.)

Variety	*IC25*		
	Dry Carrot equivalents (μg/mL)		
Commercial baby carrots	708.1	±	61.6
Commercial market carrots	391.9	±	77.3
Danvers	1321.9	±	150.0
White Satin	1203.9	±	47.3
Yellowstone	1110.3	±	233.6
Deep Purple	774.6	±	151.2
Queen Anne's Lace	257.2	±	39.9

The presence of other secondary plant metabolites known to form during cold storage of market carrots may account for the activity of baby carrots despite their low polyacetylene content. Baby carrots are lower in polyacetylenes since the compounds are in high abundance in the carrot outer cortex (*34*) which is removed during carrot processing. The presence of other secondary plant metabolites known to be present in cold stored baby and market carrots require further investigation, but is a potential health benefit of commercially available carrots held under cold storage conditions.

Purple carrots are unique due to the presence of acylated cyanidin glycosides pigments. Enriched preparations of anthocyanidin pigments possess anti-inflammatory bioactivity by reducing chemokines, chemoattractants, and cell adhesion factors (*70*). Anthocyanins reduce nitric oxide release from macrophage cells and NF-κB activation in endothelial cells (*71*, *72*). Purple carrots also contain over 40 phenolic acids (*73*) in addition to other common carrot phytochemicals such as carotenes.

A purple carrot fraction enriched in anthocyanins from LH-20 resin chromatography dose responsively reduced nitric oxide release in LPS stimulated macrophages by as much as 94% without affecting viability. Treatment with the purple carrot fraction also reduced mRNA of several LPS induced cytokines (IL-6, IL-1β) and inducible nitric oxide synthase (iNOS) in the same macrophage cell line (Figure 2). In addition, the purple carrot fraction also reduced IL-6 and TNF-α secretion in primary pig aortic endothelial cells.

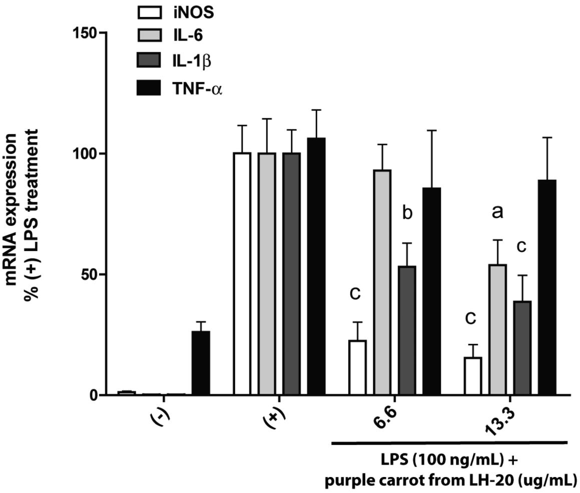

Figure 2. Purple carrot regulation of mRNA expression of inflammatory cytokines and iNOS in macrophage cells. Cells were incubated overnight in the presence of LPS and purple carrot extracts. Differences from positive control are indicated by significance (n = 6) a, P < 0.05; b, P < 0.01; c, P < 0.001. (Adapted with permission from reference (36). Copyright 2008 ACS Publications.)

Chromatographic analysis of the bioactive purple carrot extract in Figure 2 indicated the presence of various phytochemicals including anthocyanins, phenolic acids, polyacetylenes, and carotenoids. The bioactive purple carrot fraction was sub-fractionated to isolate different phytochemical groups and individual polyacetylenes from the carrot. The polyacetylene rich sub-fraction and isolated polyacetylenes are responsible for the anti-inflammatory activity as indicated by a reduction of nitrite concentration in LPS stimulated macrophage cells (Figure 3). The bioactivity of a purple carrot extract containing polyacetylenes and other phytochemicals, including other potential secondary metabolites, is less cytotoxic when compared to isolated polyacetylenes and more effective for suppressing inflammatory markers in a macrophage cell model (*55*). This indicates the importance carrots as a food with potentially additive or synergistic mixes of phytochemical classes or secondary plant metabolites that inhibit inflammatory processes more efficiently than isolated fractions of polyacetylenes.

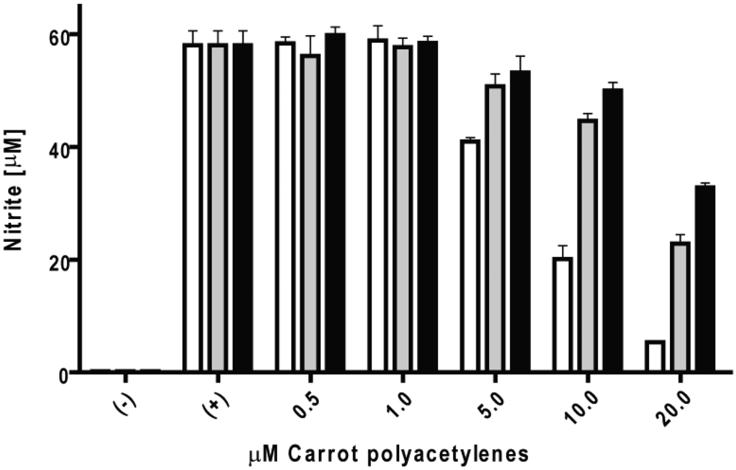

Figure 3. Bioactivity of isolated carrot polyacetylenes in LPS-induced nitric oxide production in macrophage cells. Purity of isolated polyacetylenes was > 99.5% pure by GC-MS. (n = 4). (Adapted with permission from reference (36). Copyright 2008 ACS Publications.)

The bioavailability of the polyacetylenes is another valid reason for elevating carrots status on the superfood list. Some classes of phytochemicals in foods have limited bioavailability due to low rates of absorption. This is commonly recognized for various classes of polyphenolic compounds which may have good *in vitro* support for biological activity, but low rates of absorption as intact molecules. This is the case for the extremely complex group of proanthocyanidin compounds (*74*).

Carrots contain a number of phytochemical constituents that have proven action *in vivo* and a well-researched understanding of dietary absorption. Carotenoid bioavailability from carrots is known to occur and increase with heat processing (*75*) and fat content of the diet (*76*). 5-O-caffeoylquinic acid or chlorogenic acid is the primary polyphenol in carrots and accounts for 80% of the total phenolics present (*77*) with good reported bioavailability (*78*). Purple carrot juice was shown to reduce oxidative stress, hepatic inflammation, and abdominal fat deposition in a rodent model of metabolic syndrome. The positive results were specific to the carrot juice diet and not equitable to a β-carotene diet treatment arm (*79*). Colored carrot varieties were also shown to increase liver vitamin A stores and antioxidant activity over white carrots devoid of carotenoids (*6*).

The bioavailability of the polyacetylene compounds has been investigated in an established model of colon cancer. In the model, rats fed were fed either a realistic dietary dose of carrots or an equivalent concentration of falcarinol contained in the carrot diet (35µg/g diet). As a result of both the carrot and falcarinol containing diet, the rats had a reduced tendency toward pre-cancerous lesion development. The effect of the carrot dietary interventions was greater with increasing tumor size (*57*). Additionally, carrot juice consumption (900 mL) in humans resulted in blood level concentrations of 2.5 mg/mL at 2 hours after dosing (*30*, *80*). This concentration is within the range of noted biological activity in *in vitro* models for inhibition of proliferation of cancer cells (*10*, *42*).

Conclusions

The bisacetylenic oxylipins, or polyacetylenes, are one among many secondary plant defense compounds in carrots that possess bioactivity for human health. Carrot polyacetylene compounds are potent phytoalexins capable of inhibiting numerous lipid metabolizing enzymes, and possess anti-inflammatory and anti-carcinogenic properties. Understanding unique polyacetylene sources, extractions methodologies, and mechanisms of action will provide appropriate material for further *in vivo* support. Further research on carrot secondary metabolites, outside of the range of major phytochemical components such as carotenes, will likely provide beneficial insights into the sometimes “bitter” health benefits of carrots to raise their status appropriately on the superfood list.

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Chapter 11

Nontargeted Profiling of Specialized Metabolites of *Digitalis purpurea* with a Focus on Cardiac Glycosides

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There is an urgent need to develop rapid and powerful tools for structural annotation and natural product identification to support the discovery of genes involved in specialized metabolite accumulation. In this study, nontargeted profiling of metabolites from tissues of the medicinal plant *Digitalis purpurea* was exploited. Application of liquid chromatography/time-of-flight mass spectrometry (LC-TOF MS) with multiplexed collision-induced dissociation (CID) generated molecular and fragment ion masses to support metabolite identification. Twenty-nine metabolites extracted from various tissues were annotated as steroidal glycosides, on the basis of mass measurements of pseudomolecular and fragment ions in conjunction with previous reports of steroidal glycosides in *Digitalis* tissues. The MS/MS spectra obtained by hybrid triple quadrupole-linear ion trap (QTrap) MS were complemented by accurate pseudomolecular mass measurements generated with TOF MS and fragment ion masses generated by quasi-simultaneous collision voltage. These combined methods permitted the metabolite profiling of cardiac glycosides and the tentative identification of a novel cardiac glycoside.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Introduction

The fraction of dietary constituents that have been identified or quantified is remarkably small, which makes it challenging for producers to label products accurately and for consumers and health professionals to interpret such labels. Moreover, the limited nature of available information about consumed chemicals makes it impractical to assess the risks and benefits associated with their consumption. Therefore, it is not surprising that the regulation of dietary supplements varies widely across governmental jurisdictions. A recent report by the United States Government Accountability Office (GAO) entitled "Dietary Supplements, FDA should take further action to improve oversight and consumer understanding" (*1*) indicated an urgent need to develop rapid and powerful tools for the annotation and identification of natural products contained in dietary foods and supplements. To this end, the present chapter presents an original approach for the nontargeted profiling of specialized metabolites, including steroidal glycosides, in biological samples including foods.

Steroidal Cardiac Glycosides Are Important Metabolites Present in Various Biological Samples

Steroidal glycosides represent a diverse group of secondary metabolites that include cardiac glycosides present in *Digitalis lanata* (*2*), sapogenins present in soapwort (*Saponaria officinalis*) (*3*), steroidal alkaloid glycosides present in tomato (*Solanum lycopersicum*) (*4*, *5*), and C_{21} steroidal-derivatives present in *Hoodia gordonii* (*6*). Some of these secondary metabolites possess commercial applications as medicines (*7–9*), weight loss dietary supplements (*10*), emulsifiers, and sweeteners (*11*). In particular, cardiac glycosides possess distinct therapeutic applications, and their members include the well-known pharmaceuticals digoxin and digitoxin (*12*, *13*). In the 1980s, digoxin was the fifth most commonly prescribed drug in North America (*14*). However, the narrow gap between therapeutic effect and toxicity has limited the use of cardiac glycoside-containing plants as traditional herbal medicines.

Specialized cardiac glycoside metabolites accumulate in numerous plant species, including *Beaumontia brevituba* (*15*), *Urginea maritime* (*16*, *17*), and the beautiful flowering plant *Digitalis purpurea* (*18*) (Foxglove or Lady's Glove). Cardiac glycosides from *Digitalis* plants increase the force and speed of myocardial systolic contraction and positive inotropic action for the treatment of heart disease. Inotropic effects are the common actions of drugs that increase the contractile force of cardiac muscles (*19*). The actions of *Digitalis* compounds are attributed to the inhibition of Na^+, K^+ -ATPase, a plasma membrane ATPase-powered ion pump (*20*). The intracellular Na^+ concentration increases when the enzyme is inhibited, which affects Na^+/Ca^{2+} exchange. This increase in intracellular Na^+ concentration is followed by a higher concentration of Ca^{2+}, which regulates muscle contraction by stimulating the transport of neurotransmitters across the synapses (*21*, *22*). Steroidal groups play important roles in these processes, owing to their affinity to Na^+, K^+ -ATPase. The identification of cardiotonic glycosides of the bufadienolide class in animals

(*23*) has stimulated interest in the endogenous production of cardiac glycosides in humans (*24*).

In addition to their established role in heart treatment, cardiac glycosides exhibit other potential therapeutic uses. A recent review article introduced cardiac glycosides as novel cancer therapeutic agents (*25*). Cardiac glycosides inhibit the proliferation of malignant cells, but not normal cells (*26–28*). They also sensitize human tumor cells, but not normal cells, to radiation treatment (*29–31*). Inhibition of NF-κB signaling may play a role in these pharmacological effects (*32*). First generation versions of these types of anticancer drugs are currently being tested in clinical trials (*33*, *34*).

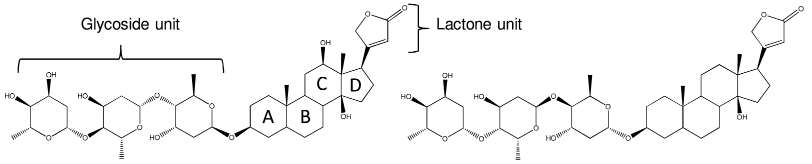
Methods for the Discovery and Characterization of Steroidal Glycosides

Discovery of novel steroidal glycosides and determination of the metabolic pathways responsible for their biosynthesis and degradation require sensitive and robust methods for profiling compounds in various plant materials and products, including botanical dietary supplements. The discovery of steroidal glycoside biosynthetic pathways can be accelerated through the measurement of related metabolites and the correlation of metabolite levels with mRNA transcript levels, which serve as measures of gene expression.

The chemical structures of cardiac glycosides, including cardenolides and the less-common bufadienolides, vary based on the type of the lactone group attached to the steroid compartment (Figure 1). For example, gitoxin is a cardenolide that contains an unsaturated γ lactone group (Figure 1a), whereas proscillaridin is a bufadienolide that contains an unsaturated δ lactone group (Figure 1f). The chemical structures also vary according to the number of different substituents (-H, -OH and $-CH_3$) and the numbers and types of glycoside units.

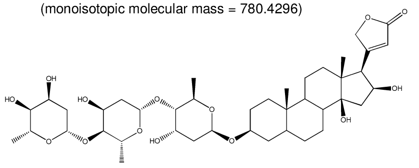
The results of an earlier report involving the feeding of [^{14}C] pregnenolone glycoside to *D. lanata* suggested that cardenolide-forming plants produce enzyme(s) capable of converting pregnenolone into cardenolides via modification of the steroidal side chain (*35*). Additional studies support the notion that cardenolides are derived from pregnane derivatives (*36*, *37*). A recent study identified *D. purpurea* P5βR2 as a novel gene involved in cardenolide biosynthesis (*38*). Glycosylation or the production of glycoside units is considered the last step in the biosynthesis of cardiac glycosides (*39*).

Liquid chromatography/mass spectrometry (LC/MS) is the methodology of choice for the profiling of steroidal glycosides, because it does not require derivatization and can be used to analyze metabolites with extensive glycosylation. Cholestane glycosides with antitumor activity were analyzed by LC-MS on a quadrupole instrument by using either electrospray ionization (ESI) or atmospheric pressure chemical ionization (*40*). Steroidal glycosides of *H. gordonii* were classified into two groups (hoodigenin A and calogenin) with electrospray ion-trap tandem MS (ESI-MS/MS) and ESI time-of-flight MS (LC/ESI-TOF MS) (*41*). The proposed MS/MS fragmentation pathway has been used to characterize sugar moieties and the aglycone of P57 in *H. gordonii* and other steroidal glycosides.

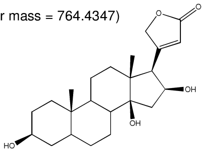


(a) Digoxin
(monoisotopic molecular mass = 780.4296)

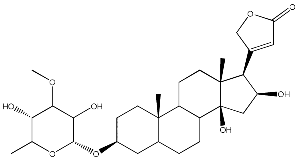
(b) Digitoxin
(monoisotopic molecular mass = 764.4347)



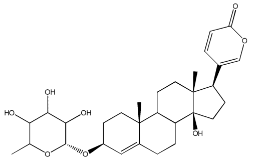
(c) Gitoxin
(monoisotopic molecular mass = 780.4296)



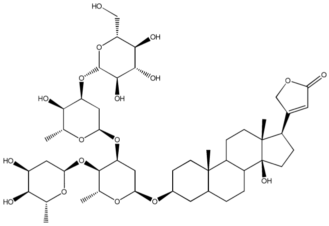
(d) Gitoxigenin
(monoisotopic molecular mass = 390.2406)



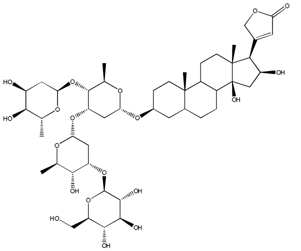
(e) Strospeside
(monoisotopic molecular mass = 550.3142)



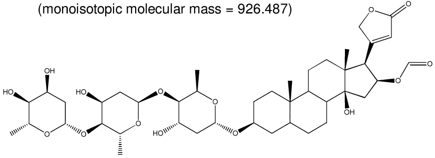
(f) Proscillaridin
(monoisotopic molecular mass = 530.2879)



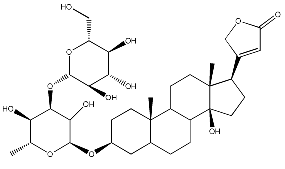
(g) Purpurea glycoside A
(monoisotopic molecular mass = 926.487)



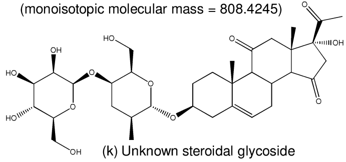
(h) Purpurea glycoside B
(monoisotopic molecular mass = 942.4824)



(i) Gitaloxin
(monoisotopic molecular mass = 808.4245)



(j) Glucodigifucoside
(monoisotopic molecular mass = 682.3564)



(k) Unknown steroidal glycoside
(monoisotopic molecular mass = 666.3251)

Figure 1. Structures of reported cardiac glycosides in Digitalis plants.

Steroidal saponins in *Yucca gloriosa* flowers (*42*), steroidal glycosides including furostanol, spirostanol, and cholestane glycosides from the leaves of *Ruscus colchicus* (*43*), and four isomers of synthesized steroidal saponins (*44*) have been analyzed by electrospray multistage (ESI-MS^n) ion-trap MS. To evaluate the absorption, tumor affinity, and metabolism of two steroidal glycosides of wildfoside C3N and wildfoside C1N in mice plasma and tumor homogenate, steroidal saponins were characterized by a triple quadrupole LC-MS system (*45*). These studies seem to have overcome the main challenges associated with the analysis of plant saponins, which typically are present in a mixture of components with similar polarities.

Various MS- and non-MS–based techniques have been employed to analyze cardiac glycosides. Two-dimensional thin-layer chromatography (TLC) was performed for the quantitative analysis of five cardiotoxins, including gitoxin and digitoxin in gastrointestinal contents (stomach, rumen, colon and cecum contents), feces, and plant material (*46*). Another TLC technique was used for the quantitative analysis of a series of cardiac glycosides (*47*). HPLC with photodiode array detection was employed in the qualitative analysis of cardiac glycosides in *D. lanata* (*48*) and healthy and phytoplasma-infected plants (*49*), and in the quantitative analysis of cardiac glycosides in *D. purpurea* (*50–53*). Mass spectrometric techniques, including LC-ESI-MS, were applied in the analysis of digoxin (*54*). Liquid chromatography triple quadrupole MS was used for the quantification of digoxin in human (*55*) and rat (*56*) plasma and the monitoring of serum digoxin (*57*). Mass spectrometry approaches led to the identification of digitalis-like cardiac glycosides as endogenous mammalian metabolites (*58*, *59*).

Novel Approach for Metabolic Profiling of Steroidal Glycosides in Biological Samples

This chapter presents an original approach for the nontargeted profiling of metabolites, including steroidal glycosides in biological samples. In particular, we describe the nontargeted metabolite profiling of specialized metabolites in various tissues of *D. purpurea*. The proposed technique relies on the quasi-simultaneous generation of mass spectra under gentle conditions that largely yield molecular mass information. Several more-energetic conditions were used to generate fragment ions, which often can provide information about molecular structure. The rapid data acquisition provided by TOF MS allowed sufficiently fast switching that was compatible with the chromatographic time scale.

Twenty-nine metabolites extracted from various tissues were annotated as steroidal glycosides, based on mass measurements of pseudomolecular and fragment ions in conjunction with previous reports of steroidal glycosides in *Digitalis* tissues. Accurate mass measurements of pseudomolecular and fragment ions obtained with LC-TOF mass spectrometry complemented the MS/MS spectra generated with LC-QTrap MS for the metabolite profiling of cardiac glycosides.

Experimental Section

Chemicals

HPLC-grade acetonitrile, isopropanol, water, and methanol were from EMD Chemicals (Gibbstown, NJ, USA). Formic acid (88%) and NH_4OH (14.8 M), digoxin (≥95.0%), digitoxin (≥98.0%), gitoxin (~95%), and gitoxigenin (≥98.0%) were from Fluka (St. Louis, MO), and gitoxin (~95%) was from Sigma (St. Louis, MO).

Preparation of Extracts and Preconcentrations

Extracts of *D. purpurea* tissues (including immature and mature leaves and fruit) were prepared by the laboratory of Professor Joseph Chappell at the University of Kentucky, and were shipped to Michigan State University. Extractions were conducted by adding 10 mL of extraction solvent (chloroform: methanol 1:1 [v/v] + 0.1% BHT and 10 µM propyl 4-hydroxybenzoate internal standard) to 1.0 g of tissue powder in a 24-mL glass vial. The suspension was shaken for 3 h on a rotary shaker. The corresponding extract was filtered with a miracloth/funnel, and filtrates were collected. The extract was concentrated 10-fold under reduced pressure with a SpeedVac (SPD131DDA, Thermo Electron Corp., Vantaa, Finland). Extracts were stored at -20 °C. No degradation of the extracts was observed at this storage temperature.

Instrumentation and Analytical Methods

LC-TOF MS Analysis

The LC-MS analyses were performed on a Shimadzu HPLC (LC-20AD pump) coupled to a Waters LCT Premier™ TOF mass spectrometer. Standard ESI was employed. Chromatographic separation was performed with an Ascentis® Express C18 Analytical HPLC column (5 cm × 0.21 cm, 2.7 µm, Supelco). The elution gradient was started at 95% solvent A (10 mM ammonium formate, adjusted to pH 2.85 with formic acid) and 5% solvent B (acetonitrile/isopropanol [1:2 v/v]) for 1 min, followed by 25% solvent A for 20 min, 5% solvent A for 5 min, 0% solvent A for 30 min, 50% solvent A for 31 min, and 95% solvent A for 37 min, at a flow rate of 0.3 mL/min. The injection volume, column temperature, and sample temperature in the autosampler were 10 µL, 40 °C, and 10 °C, respectively.

To perform multiplexed collision-induced dissociation, the cone voltage was maintained at 30 V and the aperture 1 voltage was switched to 20, 40, 60, and 80 V to induce nonselective ion fragmentation. The scan duration for each function was 0.15 s. The software used for instrumental control and data processing was MassLynx™ version 4.1.

LC-QTrap MS Analysis

The LC-QTrap MS analyses employed a 3200 QTrap triple quadrupole/linear ion-trap MS (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with binary model LC20AD HPLC (Shimadzu, Kyoto, Japan). The mass spectrometer was operated in positive and negative ionization modes, with a spray voltage of ±5.0 kV and source temperature of 500 °C. High purity nitrogen was used as the collision gas. Curtain gas, Gas 1, and Gas 2 were set at 10, 15, and 10, respectively. Enhanced MS (EMS) and Enhanced Product Ion (EPI) scans were executed with information-dependent acquisition (IDA). Chromatographic conditions and solvents were similar to the description under LC-TOF MS. Analyst software (version 1.4.2) was used for instrument control, data acquisition, and data processing.

Analysis of the Extracted Ion Chromatograph Peak Area

The extracted chromatograph (XIC) peak area for each metabolite was determined by integration and was reported with MarkerLynx™ software, version 4.1 (Waters Inc.). MarkerLynx processes the acquired chromatographic full-scan data, and provides the sample information, including peak areas and significance score. The significance score is the distance of the marker from the origin of the loadings plot, scaled to a value between 0 and 1. In MarkerLynx, the loading plot usually refers to the results of principal components analysis (PCA), which is an unsupervised multivariate statistical analysis that reduces the complexity of data variance by reducing to a two-dimensional scores plot.

Results and Discussion

Metabolite Profiling of Steroidal Cardiac Glycosides Using LC-TOF MS and LC-QTrap MS

Figure 1 presents structures of the various cardiac glycosides that have been reported in *Digitalis* plants (*60–67*). The compounds identified in the present study with LC-TOF MS (Table I) and LC-QTrap MS (Table II) differed from each other in the numbers and types of glycoside units, numbers and positions of functional groups (mainly hydroxyl groups), and types of lactone groups. The observed number of glycoside units varied from zero (in gitoxigenin) to four (in purpurea glycosides).

Metabolic Profiling with LC-TOF MS

Table I presents the identities of 11 cardiac glycosides detected on the basis of their monoisotopic masses with LC-TOF MS. Formate adducts ([M + formate]$^-$) and ammonium adducts ([M + NH_4]$^+$) were formed in the negative and positive ion modes, respectively. For each identified cardiac glycoside, Table I also shows the retention time (t_R), molecular formula, monoisotopic masses of adducts in positive and negative ionization modes, steroidal unit groups (discussed in detail in the subsection "Powerful approach for nontargeted profiling of steroidal glycosides in *D. purpurea*"), and tissue with the maximum XIC peak area. Because digoxin, digitoxin, gitoxigenin, and gitoxin were available as standards, they were identified based on retention times and monoisotopic masses of adducts and fragments.

Figure 2 shows the XICs of five cardiac glycosides in *Digitalis purpurea* fruit extract, based on *m/z* 568.323 (strospeside), 668.402 (digoxigenin-bis(digitoxoside)), 798.464 (gitoxin), 944.522 (purpurea glycoside A), and 782.470 (digitoxin). Gitoxin and digoxin, which have the same molecular mass, glycoside units, and numbers of hydroxyl groups, were well resolved by LC (t_R = 10.1 min *vs.* 8.3 min, respectively). This substantial selectivity in elution is attributed to the difference in the position and stereochemistry of one hydroxyl group in the steroidal units of gitoxin and digoxin, even though both hydroxyl groups are positioned near the lactone group.

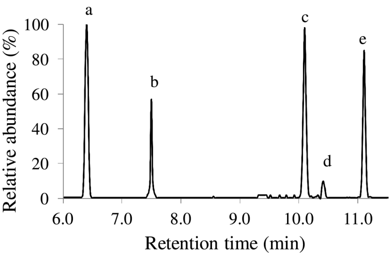


Figure 2. Extracted ion chromatogram (XIC) of the ammonium adduct of (a) strospeside (m/z 568.323), (b) digoxigenin-bis (digitoxoside) (m/z 668.402), (c) gitoxin (m/z 798.464), (d) purpurea glycoside A (m/z 944.522), and (e) digitoxin (m/z 782.470) in Digitalis fruit extract using ESI (+) LC-TOF MS.

In the data analysis of 26 *D. purpurea* samples, digoxigenin-bis(digitoxoside) and an unknown steroidal glycoside had the highest significance scores from a set of 4000 detected metabolite signals. The peak areas of metabolites with a high significance score are usually the highest peak areas of all metabolites. Cardiac glycosides were identified with the procedures described for digoxigenin-bis(digitoxoside) and an unknown steroidal glycoside (see subsection "Identification of cardiac glycosides in *D. purpurea*, including glucodigifucoside and a novel compound").

Table I. LC-TOF MS Assignments Based on an Accurate *m/z* and Multiplexed CID Fragmentation Patterns

Identified compounds	*Retention time (min)*	*Molecular formula*	*[M + HCOO]⁻ (observed)*	*[M + NH_4]⁺ (observed)*	*Steroid unit groups*[b]	*Tissue with maximum average peak area*
Digoxin[c]	8.3	$C_{41}H_{64}O_{14}$	825.43	798.46	**A**	Leaf (immature)
Digitoxin[c]	11.1	$C_{41}H_{64}O_{13}$	809.43	782.47	**B**	Fruit (immature)
Gitoxigenin[c]	7.3	$C_{23}H_{34}O_5$	435.24	408.28	**A**	Leaf (mature)
Gitoxin[c]	10.1	$C_{41}H_{64}O_{14}$	825.43	798.46	**A**	Leaf (mature)
Strospeside	6.4	$C_{30}H_{46}O_9$	595.31	568.35	**A**	Fruit (immature)
Purpurea glycoside A	10.4	$C_{47}H_{74}O_{18}$	971.48	944.52	**B**	Young leaf
Purpurea glycoside B (& isomer)	11.1 11.5	$C_{47}H_{74}O_{19}$	987.48	960.52	**A**	Young leaf
Gitaloxin	9.9	$C_{42}H_{64}O_{15}$	853.42	826.46	**D**	Leaf (mature)
Glucodigifucoside	6.5	$C_{35}H_{54}O_{13}$	727.35	700.39	**B**	Leaf (mature)
Digoxigenin-bis(digitoxoside)[i]	7.5	$C_{35}H_{54}O_{11}$	695.36	668.38	**A**	Fruit (immature)
Unknown steroidal glycoside[ii]	5.8	$C_{34}H_{50}O_{13}$	711.32	684.36	**E**	Leaf (mature)

[i] Highest and [ii] Second-highest MarkerLynx significance scores in the data analysis of 26 samples of different mature or immature tissues. [b] Refer to Figure 6 for structures and grouping rules. [c] These compounds were characterized as pure standards and in plant extracts.

Table II. LC-QTrap MS assignments based on *m/z* of adducts ions, and MS/MS EPI (- & +) fragments

Identified compounds	*Retention time (min)*	*m/z (observed)*	*Assignment*	*Polarity*	*Enhanced product ion fragments in ESI (+ and - modes)*
Digoxin[a]	8.3	825.5	$[M + HCOO^-]$	(-)	779.5, 649.6, 519.5, 475.5
		826.4	$[M + NH_4^+ + HCOOH - H_2O]$	(+)	798.4, 651.5, 521.5, 391.4
Digitoxin[a]	11.1	809.5	$[M + HCOO^-]$	(-)	763.4, 633.4, 503.4
		810.4	$[M + NH_4^+ + HCOOH - H_2O]$	(+)	782.5, 635.5, 375.6, 505.5
Gitoxigenin[a]	7.3	435.5	$[M + HCOO^-]$	(-)	389.6, 371.7, 353.6
		436.3	$[M + NH_4^+ + HCOOH - H_2O]$	(+)	408.3, 373.4, 355.4, 337.4
Gitoxin[a]	10.1	825.4	$[M + HCOO^-]$	(-)	779.5, 649.6, 519.5, 475.5
		826.4	$[M + NH_4^+ + HCOOH - H_2O]$	(+)	798.4, 651.5, 521.5, 391.4
Strospeside	6.4	595.4	$[M + HCOO^-]$	(-)	549.5, 371.3
		596.4	$[M + NH_4^+ + HCOOH - H_2O]$	(+)	
Purpurea glycoside A	10.4	971.5	$[M + HCOO^-]$	(-)	925.6, 763.4, 633.4, 503.6
		972.4	$[M + NH_4^+ + HCOOH - H_2O]$	(+)	
Purpurea glycoside B (& isomer)	11.1, 11.5	987.4	$[M + HCOO^-]$	(-)	941.6, 779.6, 649.4

Identified compounds	*Retention time (min)*	*m/z (observed)*	*Assignment*	*Polarity*	*Enhanced product ion fragments in ESI (+ and - modes)*
		988.4	[M + NH_4^+ + HCOOH − H_2O]	(+)	
Gitaloxin	9.9	853.4	[M + $HCOO^-$]	(-)	807.5, 529.4, 353.5
		854.4	[M + NH_4^+ + HCOOH − H_2O]	(+)	
Glucodigifucoside	6.5	727.4	[M + $HCOO^-$]	(-)	681.5, 519.6, 373.3
		728.4	[M + NH_4^+ + HCOOH − H_2O]	(+)	
Digoxigenin-bis(digitoxoside)	7.5	695.36	[M + $HCOO^-$]	(-)	547.3, 417.2
		696.36	[M + NH_4^+ + HCOOH − H_2O]	(+)	
Unknown cardiac glycoside	5.8	665.3	[M - H]	(-)	503.4, 341.4
		712.4	[M + NH_4^+ + HCOOH − H_2O]	(+)	

[a] These compounds were characterized as pure standards and in plant extracts.

Multiplexed ion fragmentation provides a powerful method for determining accurate molecular and fragment masses for a wide range of metabolites in a single analysis. However, its use of nonselective ion fragmentation can make it challenging to assign fragments to specific precursor ions when multiple metabolites are eluted simultaneously from the LC column. To confirm the association of fragment ion masses with specific molecular precursors, LC/MS/MS analyses were performed with a QTrap mass analyzer. Table II presents the compounds that were identified based on their *m/z* and fragmentation patterns from the EPI scan in positive and negative ion modes. In the negative ion mode, formate adducts ($[M + HCOO]^-$) were the dominant ions formed. In the positive ion mode, unusual adducts corresponding to $[M + NH_4 + HCOOH - H_2O]^+$ were identified.

The EPI mass spectrum (MS/MS, ESI (+) QTrap MS, 30 eV collision energy) of the digoxin standard at *m/z* 826.46 corresponding to $[M + NH_4 + HCOOH - H_2O]^+$ is shown in Figure 3a. The fragment ions derived from $[M + NH_4 + HCOOH - H_2O]^+$ for digoxin are also presented, and are suggestive of the formation of a formate ester adduct within the mass spectrometer ion source.

The product adduct with *m/z* of 798.46 in Figure 3b retains nitrogen from the ammonium adduct, as evidenced by the even nominal mass of the ion. Relative to the precursor ion at *m/z* 826.46, the mass difference of 28 Da is attributed to loss of CO from the formate ester. Further analysis of the lower-mass fragments suggests additional losses of carbohydrate units, eventually yielding the protonated aglycone at *m/z* 391.25. This unusual ion chemistry was only observed with the QTRAP instrument, and was not seen with the LC-TOF instrument.

The differences in the ESI ion source configurations of TOF MS and QTrap MS, which have different manufacturers, may explain the observation of $[M + NH_4]^+$ in TOF MS compared to the dominance of $[M + NH_4 + HCOOH - H_2O]^+$ in QTrap MS. A recent study by the Beauchamp group (*60*) showed that the gas-phase ionization and observed product distribution under an ambient pressure ion source depend on the configuration of the ion source and the compositions of water vapor and oxygen surrounding the source. The formation of the unusual adduct of $[M + NH_4 + HCOOH - H_2O]^+$ should caution analysts that unexpected adducts may form in the mass spectrometer ion source. Formation of such adducts may be important for compounds such as cardiac glycosides, which do not possess acidic or basic functional groups and are less likely to form protonated ions.

Identification of Cardiac Glycosides in *D. purpurea*, Including Glucodigifucoside and a Putative Novel Cardiac Glycoside

The MS/MS fragmentation of cardiac glycosides was elucidated by IDA at different collision energies. The IDA process entails the data-dependent selection of ions detected in a survey scan, followed by the generation of an MS/MS product ion spectrum of fragments derived from the selected ion.

Figure 4 demonstrates how the LC-QTrap MS and LC-TOF MS results were applied to identify cardiac glycosides. LC-QTrap MS in the negative ion mode was useful in assigning the carbohydrate portions based on cleavage of the various glycosidic bonds by collision-induced dissociation. LC-TOF MS (with multiplexed CID) was useful for confirming elemental formulas based on monoisotopic masses of ammonium adducts. In the positive ion mode, LC-TOF MS was also useful for the differentiation of steroidal units of cardiac glycosides, based on structure-characteristic fragment ion masses (see subsection "Powerful approach for nontargeted profiling of steroidal glycosides in *D. purpurea*").

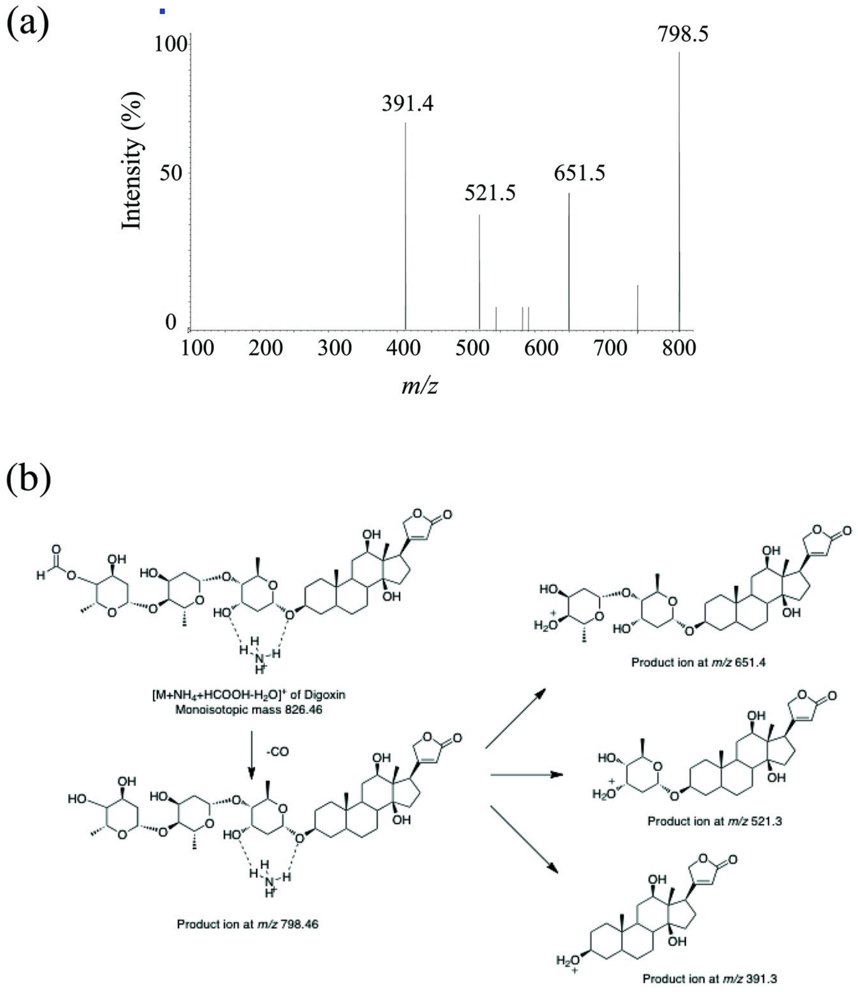


Figure 3. (a) Enhanced product ion mass spectrum (MS/MS) (ESI (+) QTrap MS, 30 eV collision energy), and (b) fragment ion assignments for digoxin adduct, [M + NH_4 + HCOOH – H_2O]$^+$ (m/z 826.4).

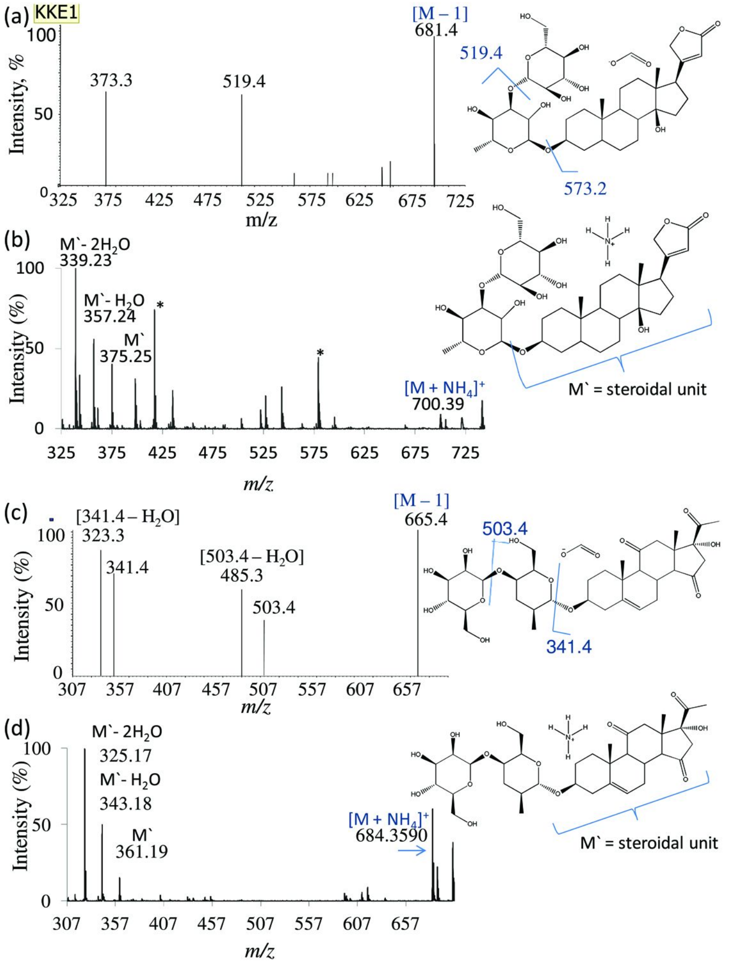


*Figure 4. Examples of procedures for the metabolite identification of cardiac glycosides: (a) enhanced product ion mass spectrum (ESI (-) QTrap MS, 80 eV collision energy) of glucodigifucoside [M + formate]$^-$ at m/z 727.4, (b) ESI (+) LC-TOF MS (at aperture 80 V) mass spectrum of glucodigifucoside, (c) enhanced product ion mass spectrum (ESI (-) QTrap MS, 80 eV collision energy) of unknown [M + formate]$^-$ at m/z 711.3, and (d) ESI (+) LC-TOF MS (at aperture 80 V) mass spectrum of unknown. *Indicates ions from coeluting metabolites.*

Figure 4a and 4b show the combined data from TOF MS and QTrap MS for the identification of glucodigifucoside in *D. purpurea*. Glucodigifucoside has only been described in a few literature reports, including mention of its presence in *Erysimum cheiranthoides* (*61*). It has been isolated from *D. lanata* and characterized with one- and two-dimensional NMR (*62*, *63*). Figure 4a shows the MS/MS product ion spectrum of the formate adduct of glucodigifucoside (*m/z* 727.4, t_R 6.5 min) generated with QTrap MS. The fragment at *m/z* 681.4 arises from loss of formic acid, and is assigned as deprotonated glucodigifucoside $[M – H]^-$. Other fragments at *m/z* 519.4 and *m/z* 373.3 result from sugar losses from deprotonated glucodigifucoside. The high collision energy mass spectrum (at aperture 1 = 80 V) of glucodigifucoside is shown in Figure 4b, which shows the ammonium adduct of this glycoside (observed *m/z* 700.3903; theoretical *m/z* 700.3903; 1.3 ppm error). Three other annotated fragments correspond to protonated glucodigifucoside aglycone and successive losses of water (*m/z* 375.2551, 357.2439, 339.2342), with mass errors of 4-7 ppm.

A similar approach was performed to annotate an unknown cardiac glycoside (Figure 4c and 4d). The MS/MS product ion spectrum (Figure 4c) of the formate adduct (*m/z* 727.4 at tR = 6.5 min) obtained with the QTrap MS showed loss of formic acid to yield the deprotonated $[M – H]^-$ at *m/z* 665.4. Fragments at *m/z* 503.4 and 485.3 represent $[M –H – sugar]^-$ and $[M –H – sugar – H_2O]^-$ ions, respectively. The fragment with *m/z* 341.4 resulted from the cleavage of two sugar units (-324 Da) from the deprotonated molecule ($[M – H]^-$). Loss of an additional water molecule yielded the observed fragment ion at *m/z* 323.3.

In Figure 4d, the mass spectrum of the unknown ammonium adduct (t_R = 5.8 min, aperture 1 = 80 V, LC-TOF MS) shows the $[M + NH_4]^+$ adduct, for a product of formula $C_{34}H_{50}O_{13}$ (observed *m/z* 684.3598, theoretical = 684.3590; mass error = 1.2 ppm). Fragments of the unknown ammonium adduct are shown in the left side of the mass spectrum representing protonated aglycone and dehydration fragments of protonated aglycone of the unknown compound (mass error ranges 4-10 ppm). Although we believe that the tentative structure is novel (Figure 4d), a metabolite with the same steroidal aglycone structure was reported in *Digitalis* plants in 1962 as digiprogenin (*64*, *65*). The positions of three keto groups, the hydroxyl group, and a double bond were drawn based on the reported structure of digiprogenin. This finding should guide further purification and characterization of the metabolite structure.

Powerful Approach for Nontargeted Profiling of Steroidal Glycosides in *D. purpurea*

A novel approach combining LC-TOF MS with multiplexed CID and information-dependent LC/MS/MS on the QTrap MS was developed for the comprehensive profiling of steroidal glycosides in biological samples. Cardiac glycosides in *D. purpurea* were profiled with multiplexed CID fragmentation to recognize steroidal substructures based on characteristic aglycone fragment masses measured with high accuracy. The multiplexed CID technology, which was first published by Robert H. Bateman at Waters MS Technologies Centre, Manchester, UK (*66*), allows the fragmentation of all of the metabolite ions eluting throughout the entire LC-TOF MS analysis. This feature functions via the rapid switching of the voltage of transit lens aperture 1 of the TOF mass analyzer.

Multiplexed CID successfully generates fragments for all metabolites, including low-abundance coeluting substances that are often missed in IDA MS/MS analyses. Chromatographic data at four different functions (i.e. collision voltages) are obtained in a single chromatographic analysis. The approach focuses on the MS of a selected part and the central unit of a steroidal glycoside structure.

In this work, the potentials of aperture 1 were switched through values of 20, 40, 60, and 80 V. Figure 5a displays the mass spectrum of the digoxin ammonium adduct in the positive mode (*m/z* 798.464) at function 4 (aperture 1 voltage of 80 V). The mass spectrum was expanded to focus on digoxin aglycone fragments. Fragments with *m/z* 373.2373, 355.2265, and 337.2162 resulted from the protonated aglycone and subsequent losses of water, and were suggestive of two hydroxyl groups.

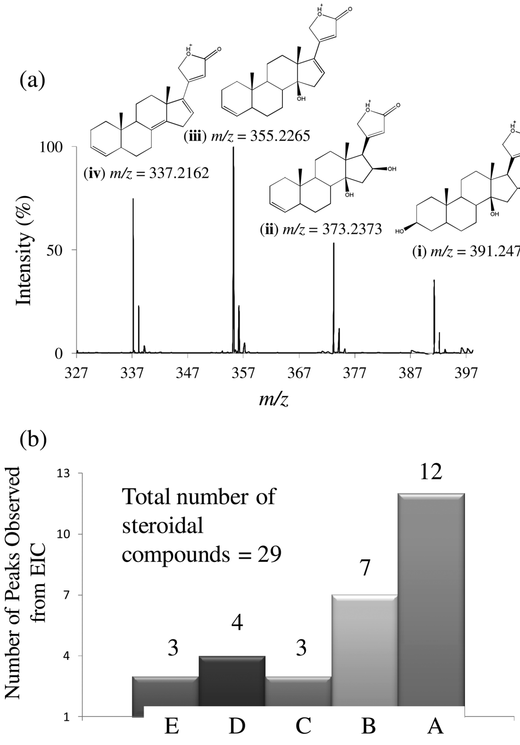


Figure 5. (a) ESI (+) LC-TOF MS (at aperture 80 V) mass spectrum of digoxin standard. i-iv represent structures in Figure 6. (b) Graph representing number of peaks observed from extracted ion chromatogram of Digitalis chromatograms at aperture 80 V, ESI (+) LC-TOF MS. A, B, C, D, and E represent steroidal structural groups drawn in Figure 6.

This approach was generalized to calculate the total number of cardiac glycosides in *D. purpurea*. Extracted ion chromatograms were generated for the *m/z* values of fragments that were characteristic of specific steroidal groups (see Figure 6) in function 4 (i.e. LC-TOF MS, at aperture 80 V) chromatograms. Figure 5b shows the number of steroidal glycosides based on the number of chromatographic peaks observed for their steroidal aglycone fragments in Figure 5a. The steroidal structural groups (A, B, C, D, and E) are designated in Figure 6.

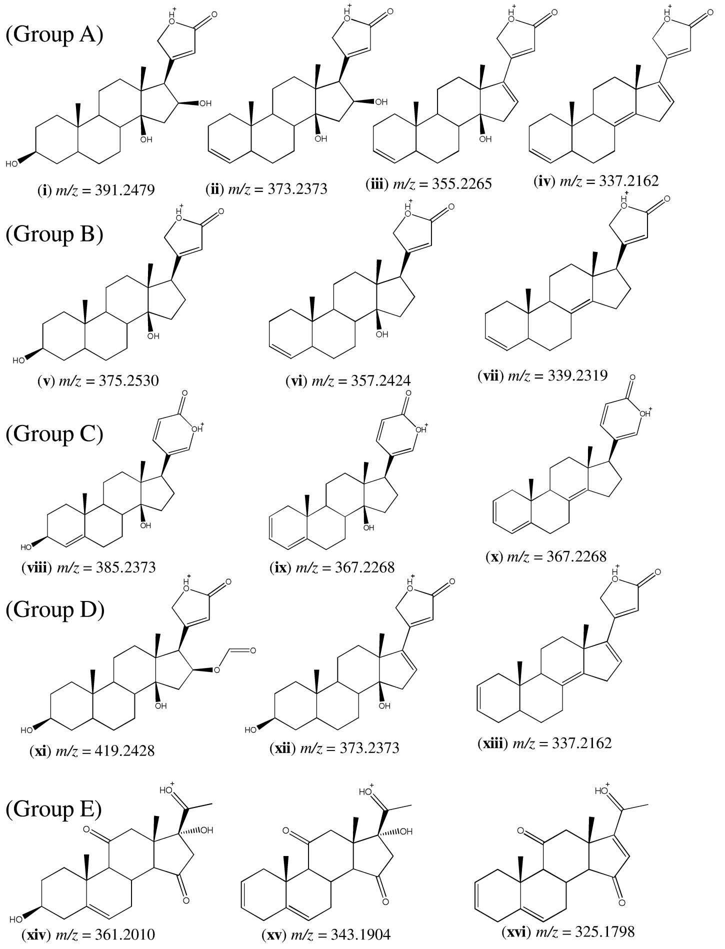

(Group A)
(i) m/z = 391.2479
(ii) m/z = 373.2373
(iii) m/z = 355.2265
(iv) m/z = 337.2162
(Group B)
(v) m/z = 375.2530
(vi) m/z = 357.2424
(vii) m/z = 339.2319
(Group C)
(viii) m/z = 385.2373
(ix) m/z = 367.2268
(x) m/z = 367.2268
(Group D)
(xi) m/z = 419.2428
(xii) m/z = 373.2373
(xiii) m/z = 337.2162
(Group E)
(xiv) m/z = 361.2010
(xv) m/z = 343.1904
(xvi) m/z = 325.1798

Figure 6. Structures of protonated steroidal unit fragments of known cardiac glycosides (LC-TOF MS, multiplexed CID at aperture 80 V). A, B, C, D, and E represent steroidal structural groups. Refer to Table I for group designations. m/z represents the monoisotopic mass of the fragment.

Based on the above analysis, we obtained 29 total putative steroidal glycosides in *D. purpurea*. The grouping is based on known steroidal units and their fragments. For example, structures in group A (i to vi) represent fragments of a steroidal aglycone structure similar to that of digoxin in Figure 5a. Examples of cardiac glycosides belonging to each group can be found in the designated column (steroid unit groups) in Table I. The possibility that the fragment masses belong to other groups of compounds is minimal, because a mass measurement accuracy of 4-7 ppm provides exquisite selectivity in distinguishing many metabolites with similar nominal masses but different elemental formulas. In addition, in Figure 5, a peak was assigned as a steroidal glycoside only if the XICs for all fragment masses occurred at a common retention time.

The discovery of plant biomarker metabolites and their concentrations in various tissues supports investigations of biochemistry in plant developmental stages (*67*). In our current study, peak areas of three selected cardiac glycosides were compared in the chromatograms of mature and immature flowers of *D. purpurea* extracts (Figure 7). Cardiac glycosides were selected based on variation in the number of glycoside units. Strospeside contained one glycoside unit, whereas purpurea glycoside A and purpurea glycoside B contained four glycoside units each. The peak areas for strospeside increased from immature to mature flower, whereas the peak areas for purpurea glycoside A and B decreased. Different factors, including enzymatic activities in mature and immature plants, could contribute to the trend in the peak areas of cardiac glycosides with different numbers of glycoside units. More replicates are needed to confirm this observation.

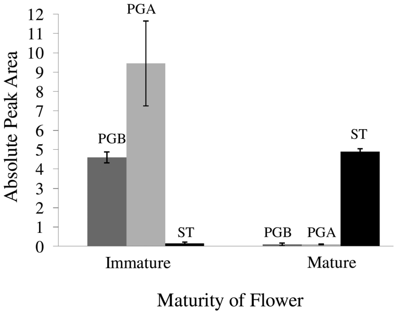


Figure 7. Peak areas of strospeside (ST, m/z 595.31), purpurea glycoside A (PGA, m/z 971.48), and purpurea glycoside B (PGB, m/z 987.48) in [M + formate]- adduct forms across mature and immature flowers of D. purpurea extracts. Peak areas were from ESI (+) LC-TOF MS results obtained with the MarkerLynx data analysis software (Waters Inc. V4.1).

Conclusions

Responding to the call for effective tools for the annotation and identification of natural products in dietary foods and supplements, and considering the pharmaceutical relevance of steroidal glycosides (*7–13*), we have developed a powerful approach for the comprehensive profiling of steroidal glycosides in biological samples. Metabolite profiling of cardiac glycosides in *D. purpurea* was accomplished with the LC-TOF and LC-QTrap MS platforms. An MS/MS scan by LC-QTrap MS revealed consecutive cleavage of sugar units. Negative and positive modes of ESI were employed for the profiling of cardiac glycoside metabolites, which are major steroidal glycosides in many plants, including *D.*

purpurea. The ability of LC-TOF MS to determine the mass, and the production of fragments by the relatively new technique of multiplexed collision-induced dissociation at high collision voltage, enriched and complemented the LC-QTrap MS cardiac glycoside metabolite profiling and enabled novel cardiac glycoside identification. We anticipate that these experimental protocols can be readily adapted to screen for unanticipated (and anticipated) natural products in a variety of dietary constituents, including natural product supplements.

Acknowledgments

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Chapter 12

Mediterranean Wild Plants As Useful Sources of Potential Natural Food Additives

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There is a growing interest in the search of new natural products to be used as additives, instead of synthetic compounds, in preserving flavour, improving taste and appearance and preventing food and drinks from mouldy or stale.

Mediterranean area is known to be an hot spot of a very high plant species biodiversity and, for its strong environmental conditions (e.g. drought stress), a potential source of wide phytochemical diversity. In this context and in order to discover new bioactive compounds, we carried out phytochemical analysis of Mediterranean wild plants species.

Crude extracts and pure metabolites have been tested by different antioxidant and radical scavenging bioassays as well as antiproliferative tests on stabilized cell lines. In order to propose the use of such plant complexes in industrially processed foods, an overall toxicity approach has been adopted considering aquatic toxicity testing, mutagenesis, genotoxicity and endocrine disrupting activity.

In the aim to identify the main metabolites responsible for the observed bioactivity, the metabolic profiling of crude extract have been performed by 1D and 2D NMR experiments.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Introduction

Plants contain active constituents that work harmoniously together, in a game of synergy and antagonisms, and are able to produce a biological effect other than the individual active constituents. The plant complex is responsible for the plant healthy properties, which can then be different from those of one or more of its components individually taken.

Among the beneficial properties of plants there is an increasing interest towards antioxidant capacity: several secondary metabolites seem to be involved in the regulation of redox homeostasis of cells.

Detrimental effects of oxidative stress on cell structures are well known. Free radicals have been demonstrated to be one of the causes of several diseases, including neurodegenerative processes, cardiovascular disorders and cancer (*1–4*). Life style, cigarettes, radiations, environmental pollution, etc. could change the physiological redox-homeostasis among free radicals and endogen antioxidant: the alteration of the free radicals amount in cells causes the alteration of biomolecules chemical structure and, consequently, the cell metabolism (*5*, *6*).

Free radicals are also determining factors of food spoiling as their production during the preparation and storage of food decreases their shelf-life, leading to economic damages and representing a risk for human health. In order to delay oxidation processes, exogenous antioxidants are generally added to food during processing and storage (*7*, *8*). Many substances with antioxidant activity occur naturally, but a variety of synthetic forms have also been developed (e.g. BHT, BHA, propyl gallate) although some evidences show that the oxidative features and/or metabolites of BHA and BHT may contribute to carcinogenicity or tumorigenicity (*9*).

In general plant substances not identified as nutrients but able to promote health and prevent diseases are called phytochemicals. They are responsible for a wide range of pharmacological effects such as antioxidative, anti-inflammatory, estrogenic/antiestrogenic, antimutagenic, antigenotoxic in animal systems (*10–14*). The discovery of new antioxidant molecules from natural sources, to be used in alternative to synthetic compounds, is a promising goal. Potential candidates have been already isolated and characterized from many plant foods (quercetin, resveratrol, ellagic acid, chlorogenic acid, etc), fruit and vegetable wastes (*15*, *16*), but also from some not edible wild plants (*17*). The last sources have been few explored in relation to the high number of wild plant species richness in the world.

In this context, wild plants species of mediterranean region could represent a promising source of potential food additives. In fact, mediterranean area is known to be a hot spot of a very high plant species biodiversity (*18*). Due to the adverse environmental conditions (e.g. drought stress), characteristic of this area, plants developed wide adaptative strategies and, among them, the production of secondary metabolites represents one of the most sophisticated communication mechanisms. As consequence the improvement of the chemodiversity makes these plants potential sources of new useful compounds (*19*). Therefore, in order to identify new bioactive compounds, we carried out phytochemical analysis of a number of selected steno and euri- Mediterranean wild plants species. Several new metabolites have been isolated and their structural elucidation performed on the basis of their spectroscopic features, specially 1D and 2D NMR experiments (*20*, *21*).

In the aim to propose bioactive plant complexes as potential food additives, crude extracts of Mediterranean wild plants have been tested using different antioxidant and radical scavenging bioassays as well as antiproliferative tests on HepG2 cell line. Furthermore, attention has been focused on the estrogenic and genotoxic/antigenotoxic effects of the chosen extracts in bacterial and yeast systems. In the case of the use of these extracts as active substances in dietary supplements it was interesting to determine their potential impact in the aquatic environment. For this reason the acute and chronic ecotoxicity on aquatic organisms has been detected. The metabolic profiling has been determined by using quantitative NMR analysis of the crude extracts.

Sampling and Analytical Methods

Plants of *Carex distachya* Desf. (Cyperaceae), *Rosmarinus officinalis* L. (Lamiaceae), *Teucrium chamaedrys* (L.) (Lamiaceae), *Teucrium polium* L. (Lamiaceae), *Petrorhagia velutina* (Guss.) Ball. et Heyw. (Caryophyllaceae), *Myrtus communis* L. (Myrtaceae) and *Arbutus unedo* L. (Ericaceae) were collected at “Castel Volturno” Nature Reserve (Southern Italy), a flat coastal area with a maximum elevation of 9 m above the sea level and characterized by stabilized dunes of alluvial deposits and loose siliceous-calcareous sand. The climate is typically Mediterranean with precipitations mostly occurring in autumn and winter with a drought period in summer. The soil is characterized by homogeneous sand with 97.1% sand, 1.25% loam, 1.6% clay, poor in organic matter and nutrients (*22*). The landscape is dominated by a low Mediterranean macchia of mixed shrub and scattered herbaceous community. Voucher specimens have been deposited at the Herbarium of the Dipartimento di Scienze della Vita of the Second University of Naples.

Plant samples were suitably reduced to fragments and lyophilized. The obtained freeze-dried powders were extracted by sonication for two hours in methanol. After removal of the solvent, we obtained dried crude extracts. The amount of total phenols in crude plant extracts was determined according to the Folin-Ciocalteu procedure reported by Kähkönen et al. (*23*).

The DPPH radical scavenging capacity of crude plant extracts was measured according to the method of Brand-Williams et al. (*24*). Determination of ABTS$^{\bullet+}$ scavenging capacity was estimated according to the method of Gallati (*25*). The determination of thiobarbituric acid (TBA) reactive species was performed by the method reported by Sroka & Cisowski (*26*), with modifications. The reducing power was determined according to the method of Oyaizu (*27*) with some modifications.

Antiproliferative activity against human hepatoblastoma cells (HepG2) was evaluated by the MTT test that allows the assessment of cell viability by determining the levels of activity of mitochondrial dehydrogenases toward 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (*28*).

The toxic potential was measured on the rotifer *Brachionus calyciflorus* and the crustacean *Thamnocephalus platyurus*, two primary consumers in the aquatic chain. The endpoint of the acute tests was mortality (*29*) while the chronic test was based on a population growth inhibition in 48 h of exposure (*30*).

Genotoxicity and antigenotoxicity were determined by the SOS Chromotest, a quantitative bacterial colorimetric assay performed on *Escherichia coli* PQ37, based on the detection of damage to DNA measured through the SOS DNA repair system (*31*). The estrogenic activity was determined using the YES-test, an estrogen-inducible yeast-screening bioassay (*32*). This method employs the yeast strain RMY326 of the *Saccharomyces cerevisiae*, transfected with a human a-estrogen receptor and an estrogen-responsible element linked to the reporter gene lac-Z, encoding the enzyme β-galactosidase, which is used to measure the receptors activity..

Metabolic profile of crude methanol extracts was obtained by dissolving 40.0 mg of each investigated plant extract in 1.0 mL of a mixture of phosphate buffer (pH 6.0, 90 mM) in D_2O (containing the internal standard) and methanol-d_4 (1:1) and analyzed by NMR. Trimethylsilylpropionic acid sodium salt (TSP, 0.01%, w/v) was used as internal standard. The metabolites in the extracts have been identified on the basis of 1D and 2D NMR experiments (COSY, TOCSY, HSQC, HMBC, J resolved).

Carex distachya Desf.

Carex distachya Desf. (Cyperaceae) is an hemicriptophytes densely caespitose plant. The genus *Carex* is commonly known as sedges and it is the most species-rich genus in the family with a species number ranging from about 1100 to almost 2000 (*33*) and a global distribution in different habitats of the world. *Carex distachya* Desf. is a steno-mediterranean species known with the Italian name "*carice mediterranea*" and characterized by stems of 10-45 cm height with blackish-brown basal sheaths and by leaves 1-2 mm wide, flat, basal and equaling the stems. This species is an annual herbaceous plant flowering from April to June and leaving in dry places of *Quercus ilex* woodland and mediterranean *macchia* from 0 to 100 m a.s.l. (*34*, *35*) . Species of *Carex* are often important to preserve moisture and are used as forage for livestock and for herbivorous wildlife. Many species of *Carex* occurring in eastern Canada, especially the long-rhizomatous ones, are of high forage value (*36*).

Plants of the *Carex* genus are characterized by the production of stilbene derivatives (*37*). The phytochemical study of *C. distachya* led to the isolation of new metabolites, named carexanes, characterized by a tetracyclic skeleton arising from a cyclization of prenyl stilbenoid precursors (*38*).The isolation and the characterization of eleven tetracyclic structures have been reported (*39*), together with further five tricyclic derivatives (*40*) and distachyasin (*41*), a carexane metabolite with high antioxidative properties, comparable to those showed by the ascorbic acid (*42*). Other abundant stilbenoids identified in *C. distachya* were resveratrol 3,5-*bis*-glucopyranoside 3,5-*bis*-*O*-β-D-glucopyranosyloxy-3′-methoxy-*trans*-stilben-4′-ol, and pallidol, a symmetric resveratrol dimer, present in both glucoside and aglycone forms (*42*). Bioactive flavonoids, flavolignans, lignans and terpenes have also been reported from this species (*43*), as well as further unusual metabolites such as dibenzoxazepinones (*44*) and feruloyl monoglyceride macrolactones (*45*).

The bioassay results of leaf and root extracts of *C. distachya* are reported in Figure 1.

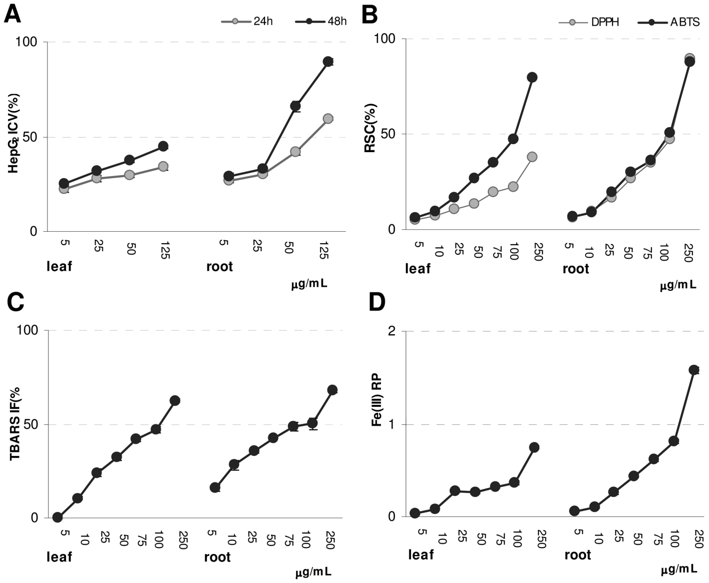


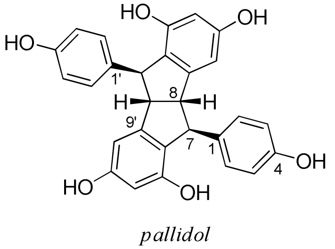
Figure 1. Bioactivity of leaf and root extracts of C. distachya. A=HepG2 Cell Inhibition Viability; B=Radical Scavenging Capacity; C=TBARS Inhibition Formation; D=Fe(III) Reducing Power. Data are reported as percentage vs blank±SD (graphs A-C) or as increase of absorbance vs blank ±SD (graph D).

The evaluation of HepG2 cell survival, carried out by MTT reduction assay, evidenced that root extract was much more active than the leaf one. The cytotoxic activity of the root extract became massive after 48 hours of exposure exhibiting an ID_{50} (half maximal inhibitory dose) value equal to 8.0 μg/mL. Both the extracts scavenged, in a dose-dependent manner, the probes $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. The root extract exercised an effective and similar antiradical capacity *vs* both the target radicals. Analogously it was able to markedly reduce Fe(III) to Fe(II). As evidenced by TBARS method, epigean and ipogean components inhibited the synthesis of lipoperoxidation secondary products in a comparable way, although the root extract was already active at the lowest tested dose. Total phenol content data, evaluated by FCR method, confirmed antioxidant data; the root extract contained 78.5 mg gallic acid equivalent (GAE) per gram of dry material (DM) whereas the leaf extract had a phenol amount equal to 70.7 mg GAE/gDM.

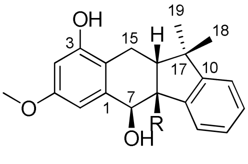
Both root and leaf extracts did not show any genotoxicity at the SOS Chromotest until the maximum concentration tested equal to 250 μg/mL.The extracts were then pre-incubated at 20, 100 and 250 μg/mL with 4-nitroquinoline-N-oxide (1 μg/mL), a standard genotoxin and again subjected to the SOS Chromotest to evaluate the possible antigenotoxic effect of these matrices. The results show a clear dose-response relationship with a maximum reduction (61%) for the leaves and 35% for the roots at the highest concentration tested suggesting that the presence of antioxidants and other bioactive compounds is associated with the decrease of the genotoxin activity. A different behaviour was found between roots and leaves for the endocrine interference activity. In fact, roots were negative at the YES test while the leaves extract showed a weak activity with a Relative Inductive Efficiency (RIE) of the expression of β-galactosidase compared to E_2 (RIE = 100%) equal to 46% at 10 μg/mL. The aquatic toxicity testing of root extracts evidenced an acute median lethal concentration for the rotifers of 46.5 and 124 μg/mL for the crustaceans. Concentrations of one or two order of magnitude lower determined a chronic impact on rotifers. Differently, the leaf extracts did not show any acute toxicity until 250 μg/mL for rotifers and crustaceans while a concentration of 7.3 μg/mL inhibited the reproduction of rotifers (chronic effect).

In the aim to investigate the main classes of secondary metabolites responsible for the observed biological effects, the metabolic profiling by 1D and 2D NMR spectroscopy has been carried out. The ^{1}H NMR spectrum of the leaf extract showed, besides the abundant saccharidic signals, two doublets at δ 6.93 and 6.71 and two singlets at δ 6.61 and 6.17. These values suggested the presence of pallidol. To confirm this hypothesys, 2D NMR experiments were planed and, in particular, the HMBC experiment evidenced heterocorrelations between the H-2/H-6 doublet protons at δ 6.93 and C-1, C-6/C-2, C-4 aromatic and C-7 and C-8 benzylic carbons at δ 54.2 and 60.9, respectively. C-1, C-2/C-6 carbons, at δ 138.2 and 128.2, showed cross peaks with the protons at δ 3.56 (H-7) and 4.44 (H-8). Furthermore both these protons heterocorrelated with the mutual carbons, as well as with the C-9 and C-10 carbons at δ 149.9 and 123.1.

In the upfield region of the spectrum diagnostic correlations, indicating the presence of carexanes, were evident. The two signals at δ 1.25 and 1.32, due to the H-18 and H-19 methyls, showed clear correlations with the C-14, C-16, C-17, C-19 and C-18 carbons, as reported in table 1, indicating the presence of carexane A. Minor analogous correlations, regarding the methyls at δ 0.67 and 1.39, suggested the presence of carexane B in the extract.

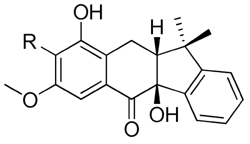


pallidol

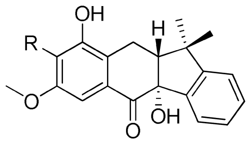


carexane A

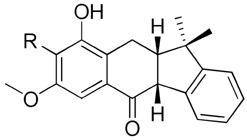
The ^{1}H NMR of root extract showed in the aromatic region signals attributable to carexanes with two or three oxygenated functions in the A-ring. In fact doublets ranging from 6.10 to 6.20 and from 6.30 to 6.35 ppm, due to the H-4 and H-6 protons, as well as singlets at δ 7.05-7.15, due to the H-6 protons in the 4-hydroxy derivatives, were present. The identification of the main carexanes was performed on the basis of the correlations between the H-18 and H-19 methyls and the C-16, C-17 and C-10 carbons as reported in table 1. The most representative carexanes present in the extract are characterized by the presence of a methoxyl and a hydroxyl on the A-ring while the 4-hydroxy derivatives are less abundant. Carexane H is the sole tricyclic metabolite detected in the root extract.



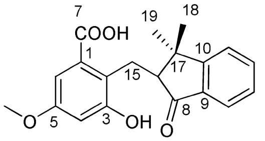
carexane B R = H
carexane F R = OH



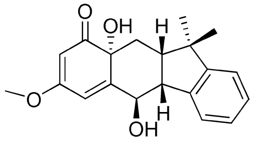
carexane C R = H
carexane G R = OH



carexane D R = H
carexane E R = OH



carexane H



distachyasin

Table 1. Diagnostic correlations, involving H-18 and H-19 methyls of carexanes, evidenced in the HMBC experiment

	^{1}H (δ)		*^{13}C (δ)*				
	H18	*H19*	*C10*	*C16*	*C17*	*C18*	*C19*
carexane A	1.25	1.32	153.3	49.8	45.6	24.4	30.6
carexane B/F	0.67	1.39	152.2	54.8	46.0	26.9	29.2
carexane C/G	1.38	1.38	153.5	58.4	43.9	27.5	27.5
carexane D/E	1.07	1.16	153.2	50.9	47.0	28.1	24.0
carexane H	1.19	1.20	164.1	58.7	43.5	28.0	28.0
distachyasin	1.22	1.00	152.2	47.0	44.9	25.0	30.5

Pallidol was first reported from *Cissus pallida* (*46*), but it has been widely found in *Vitis* spp plants and in wines (*47*, *48*). It shows antioxidant properties stronger than those found in resveratrol (*49*) and strong quenching effects on singlet oxygen at very low concentrations (*50*). Recent studies demonstrated a neuroprotective activity of resveratrol derivatives by inhibiting β-amyloid peptide aggregation. (*51*). Carexanes from *C. distachya* have been only reported to possess allelopathic activity against high plants (*20*).

Teucrium chamaedrys (L.)

Teucrium chamaedrys L. (Lamiaceae) is a rhizomatous dwarf shrub with annual flowering stems 5-50 cm high. The genus *Teucrium* includes approximately 260 species of herbs and subshrubs, with rhizomes or stolons, worldwide distributed and particularly abundant in the Mediterranean region. *Teucrium chamaedrys* L. is a perennial evergreen euri-mediterranean species characterized by a high variability of leaf and hair morphology with numerous subspecies recognized according to the environmental site conditions. The species grows in different types of habitat, from open *Quercus* woodland to dry grassland and rocky slopes ranging from 0 to 1700 m a.s.l (*34*, *35*).

Teucrium species are rich in essential oils. They are valued as ornamental plants and pollen source, and some species have culinary and/or medicinal value.

The genus *Teucrium* is the most abundant source of furanic neo-clerodane diterpenes that have attracted interest on account of their activity against some economically important lepidopteron, coleopteran and orthopteran pests (*52*). Several neo-clerodane diterpenes and phenylethanoid glycosides have been isolated from *T. chamaedrys* (*21*, *53*, *54*). The structural characterization of three new nor-*neo*-clerodane glucosides, named chamaedryosides A–C have been reported for *T. chamaedrys* collected in Mediterranean *macchia* (*55*). Besides phenylethanoid glycosides, new iridoid glycosides have been also reported from this species (*56*).

The bioassay results of leaf and root extracts of *T. chamaedrys* are reported in Figure 2.

Both the methanolic extracts from *T. chamaedrys* showed a weak inhibitory effect on HepG2 cell viability. The root extract was responsible for an activity more marked than the leaf one. While the leaf extract exercised a similar efficacy at both the exposure times considered, the root extract determined an increased dose-response and time-dependent activity. Both the extracts scavenged massively the DPPH radical and the ABTS cation radical, realising a their complete conversion to the corresponding reduced forms at highest tested dose, and inhibited the lipoperoxidative processes. The formation of TBA reactive species was strongly decreased; the leaf extract showed an ID_{50} value of 30.1 μg/mL whereas the root extract displayed an ID_{50} value of 16.7 μg/mL. The root extract showed a lower Fe(III) reducing power than the leaves. Total phenol, expressed as gallic acid equivalent, showed that leaf and root extracts had a high phenolic content of 80.1 and 84.1 mg/g GAE, respectively.

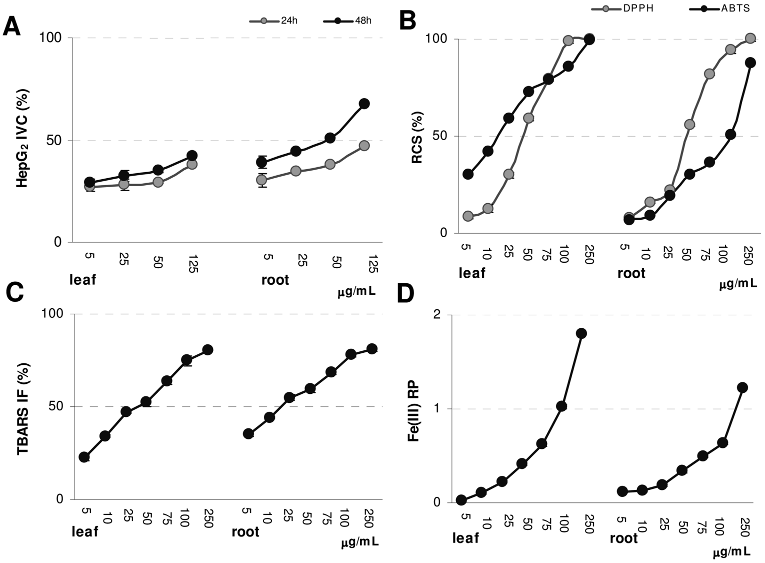


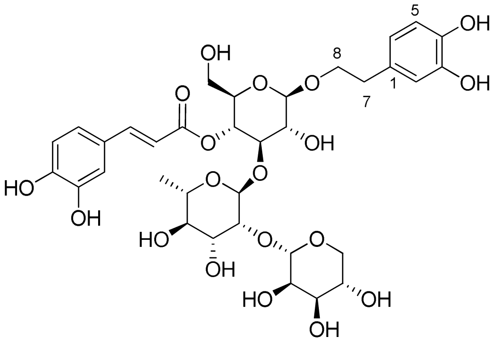
Figure 2. Bioactivity of leaf and root extracts of T. chamaedrys. A=HepG2 Cell Inhibition Viability; B=Radical Scavenging Capacity; C=TBARS Inhibition Formation; D=Fe(III) Reducing Power. Data are reported as percentage vs blank±SD (graphs A-C) or as increase of absorbance vs blank ±SD (graph D).

Both root and leaf extracts did not show any genotoxicity at the SOS Chromotest until the maximum concentration tested equal to 250 µg/mL.The extracts, pre-incubated with 4-nitroquinoline-N-oxide and subjected to the SOS Chromotest did not show any antigenotoxic effect. No endocrine interference activity was found for the root extract while the leaves were among the extracts showing the highest activity with a Relative Inductive Efficiency of 66% at 2.5 µg/mL when compared to 17β-estradiol. Both root and leaf extracts did not show any acute aquatic toxicity for rotifers and crustaceans while concentrations of 0.17 and 0.53 µg/mL, were sufficient to determine a chronic effect in the rotifers for the leaf and root extracts, respectively.

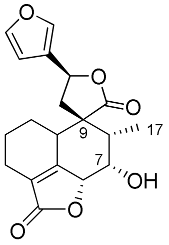
The ^{1}H NMR of the leaf extract indicated that teucrioside was the main component. The presence of its hydroxytyrosol moiety was evidenced by the presence of a triplet at δ 2.83, due to the H-7 protons, showing heterocorrelations, in the HMBC experiment, with the C-1, C-2, C-6, and C-8 carbons. This latter showed cross peaks with the anomeric glucose proton at δ 4.45. Further correlations agreed with the presence of a caffeoyl moiety bound to the C-4' carbon of glucose. Furthemore the presence, in the ^{1}H-NMR spectrum, of triplets ranging from 5.45 to 5.82 ppm correlating with carbons at δ 140.1, 124.4 and 109.0, and assigned to those of a furane ring, allowed to evidence the presence of neoclerodanes in the leaf extract. In particular, the signal at δ 1.21, corelated with the carbon at δ 13.8 (C-17 methyl), showed cross peaks with the carbons at δ 57.4, 38.2 and 72.3, attributed to the C-9, C-8 and C-7 carbons of teucrin A.

Further diterpenes signals were also evident, but the ambiguous correlations did not allow the identification of the metabolites.

The NMR data of root extract exclusively showed signals attributable to teucrioside. No signals of clerodane or other secondary metabolites have been detected.



teucrioside



teucrin A

The antioxidant capacity in cell-free systems of teucrioside was evaluated by measuring its strong capabilities to inhibit the synthesis of thiobarbituric acid reactive species in assay media using as oxidable substrates a vegetable fat, the pentose sugar 2-deoxyribose and the hydrosoluble bovine serum albumin to prevent oxidative damage (*56*). This metabolite has been shown to affect the growth and/or viability of several kinds of cancer cells (*57*). The ortho-dihydroxy aromatic ring of the hydroxytyrosol moiety is necessary for its cytotoxic and cytostatic activities.

Teucrium polium L.

Teucrium polium L. (Lamiaceae) is a dwarf subshrub with a stem 6-45 cm high and covered with greenish branched hair, often densely present, and with a pleasant aromatic smell. For general characteristics of the genus see section of *Teucrium chamaedrys* of this chapter. *T. polium* is a steno-mediterranean species characterized by creeping stems with ascending branches growing on dry places of stabilized sand dunes, dry pasture and low open macchia from 0 to 900 m a.s.l. This species shows variable morphological features with different subspecies occuring on account of the environmental site conditions (*34*, *35*). Its flowers are small, ranging from pink to white. Leaves are used in cooking and for medicinal purposes, particularly for the treatment of stomach ailments.

Teucrium polium is a medicinal plant reported to have hypolipidemic (*58*), hypoglycaemic (*59*, *60*), anti nociceptive (*61*) and anti-inflammatory (*60*) effects, but few adverse effects of *T. polium* have been described indicating the relatively safe nature of this medicinal herb (*62*, *63*).

In a previous phytochemical study on the aerial parts of *T. polium* two known phenylethanoid glycosides and two *neo*-clerodane diterpenes, teulolins A and B have been reported (*64*). Furthermore, the iridoid glycoside teucardoside, the flavonoids salvigenin and cirsiliol (*63*) and two further neo-clerodane diterpenes auropolin and capitanin (*65*) have been isolated from this plant. Sharififar et al. (*66*) reported the isolation, the structural elucidation and antioxidant evaluation of four flavonoids: rutin, apigenin, 3’,6-dimethoxyapigenin and 4’,7-dimethoxyapigenin from the aerial parts of *T. polium*. Finally, Fiorentino et al. (*67*) reported six new 17(15→16)-*abeo*-abietanes, along withfirther seven already known analogouses, as constituent of *T. polium* roots.

The bioassay results of leaf and root extracts of *T. polium* are reported in Figure 3. Although the HepG2 cell survival data were comparable to those showed from *T. chamaedrys* components, the antioxidant activity was not equally marked. Only DPPH method, with its overestimation limits, supplied a similar result. Leaf extract contained a total phenol content (64.8 mg GAE/g DM) slightly lower than root extract (77.0 mg GAE /g DM).

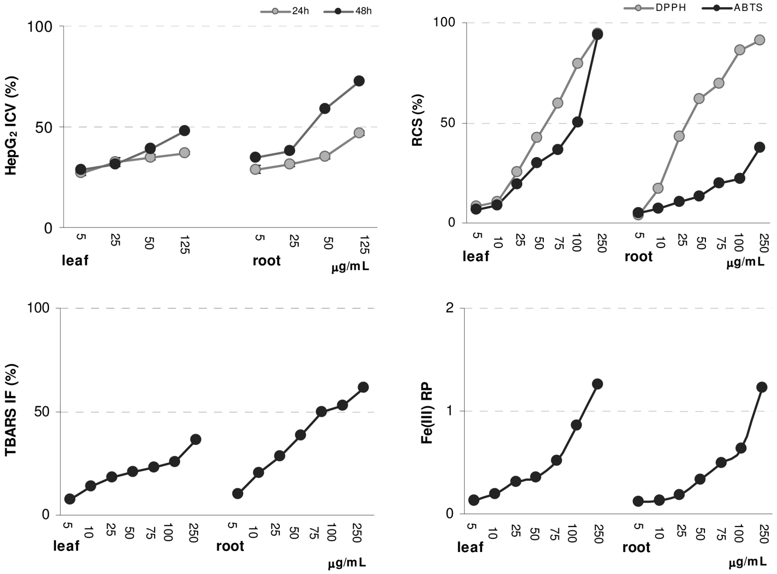
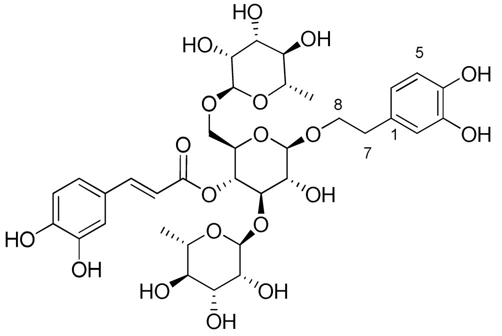


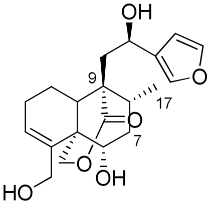
Figure 3. Bioactivity of leaf and root extracts of T. polium. A=HepG$_2$ Cell Inhibition Viability; B=Radical Scavenging Capacity; C=TBARS Inhibition Formation; D=Fe(III) Reducing Power. Data are reported as percentage vs blank±SD (graphs A-C) or as increase of absorbance vs blank ±SD (graph D).

T. polium root and leaf extracts were not genotoxic until the maximum concentration tested (250 µg/mL). At the antigenotoxicity testing both extracts showed a weak effect at the concentration of 100 µg/mL. Differently by *T. chamaedrys*, the leaf extract did not show any endocrine interference activity while the root extract was weakly positive with a maximum capability to induce the expression of β-galactosidase, in comparison to E$_2$ (RIE = 100%), of 49% at the concentration of 10 µg/mL. No acute toxicity of the root and leaf extracts was detected for rotifers and crustaceans but, in the long term exposure, the reproduction of rotifers showed a median effect concentration (EC50) of 0.49 μg/mL for the root extract and 0.31 μg/mL for the leaf extract.

As *T. chamaedrys*, also in the methanol extract of *T. polium* prevail the phenylethanoid glycosides. In fact, the main proton signals in the ^{1}H-NMR spectrum could be assigned to the catechol rings of caffeoyl and hydroxytyrosol moieties. The presence of two doublet methyls at δ 1.21 and 1.06 suggested the presence of poliumoside and the correlations showed in the HMBC experiment confirmed this hypothesis. The HMBC experiment also revealed the presence of a clerodane diterpene. In fact, the correlation between the H-17 methyl at δ 0.77 and the C-7, C-8 and C-9 carbons at δ 35.1, 28.2 and 48.0, respectively, agreed with the presence of Teulamifin B, presiously isolated from *T. lamifolium* and *T. polium* (*68*).



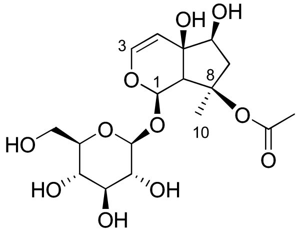
poliumoside



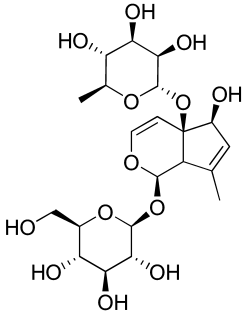
teulamifin B

Furthermore, a mixture of neoclerodanes having a spiro-lactone function was also detectable in the heterocorrelation NMR experiment.

The root extract of this plant is characterized by the presence of poliumoside, detected in greater quantities than in the leaf extract. Moreover, the presence of singlets, ranging from 5.9 to 6.1 ppm, indicated the presence of iridoids. In particular the signal at δ 6.04, attributed to the H-1 proton of iridoid aglycone, correlated in the HMBC experiment with the anomeric carbon of a glucose moiety, and the C-3 and C-5 carbons at δ 144.0 and 73.8, respectively. The H-3 proton at δ 6.42 showed cross peaks with the C-1, C-4 and C-5 carbons at δ 94.0, 106.2 and 73.8. Furthermore, the observed correlations between the H-10 methyl at δ 1.52 and the C-9 carbon at δ 52.0 and the carboxyl carbon of an acetyl moiety agreed with the presence of 8-*O*-acetylarpagid, already reported as constituent of *T. yemense* together with teucerdosid (*69*). This latter compound was also detected in the root extract of *T. polium*. In fact the H-1 proton at δ 5.92 correlated with the anomeric carbon of glucose and with the C-3 and C-5 carbons. The quaternary carbon at δ 77.2 correlated with the anomeric proton of a rhamnose unit at δ 5.40. Finally, the methyl at δ 2.29 showed cross peaks with the olefin C-7 and C-8 carbons at δ 128.3 and 176.2 according with a α,β unsaturated ketone moiety, as reported for teucardosid.



8-O-acetylarpagid



teucardosid

Poliumoside, as well as other phenylethanoid glycosides, was shown to have a wide range of biological properties, including antioxidant power (*70*), radical scavenging activity (*71*), and *in vitro* neuroprotective capacity (*72*). In particular, this phenylethanoid glycoside significantly attenuated glutamate-induced neurotoxicity at concentrations ranging from 0.1 to 10 μM. Iridoids display an interesting spectrum of biological activity such as anti-inflammatory (*73*, *74*).

Petrorhagia velutina (Guss.) Ball. et Heyw

Petrorhagia velutina (Guss.) P. W. Ball et Heywood (Caryophyllaceae) is an annual herbaceous plant up to 30 cm high, with a characteristic densely glandular-tomentose stem. The *Petrorhagia* genus includes small, annual and perennial plants mostly native to Eurasia, but some species, introduced to other continents, are worldwide distributed. It is low-growing with wiry stems and narrow, grass-like leaves. The flowers are small and in clusters similar to members of the genus *Dianthus* with pink, lilac, or white color. *Petrorhagia velutina* is a sud-mediterranean species characteristic of dry uncultivated site from 0-600 m a.s.l. and flowering from May to July (*34*, *35*).

Several secondary metabolites have been reported from this plant. Flavonoids *C*-glycosides having antiproliferative activity (*75*) were isolated from the leaves of *Petrorhagia velutina*. Besides these compounds, cinnamoyl glucose esters (*76*) were also isolated, together with phytotoxic chlorophyll derivatives (*77*).

The changes in HepG2 cell growth and proliferation in response to *P. velutina* extract treatment were evaluated by MTT assay. The ID_{50} value was calculated to be 14.8 μg/mL after 48 hours treatment. With regard to the extract antioxidative efficacy, we observed a positive, almost linear, correlation between activity and doses used in the various tests performed. The Fe(III) reducing effectiveness was weak even at the highest tested doses. Total phenol content was estimated equal to 79.3 mg GAE/g DM.

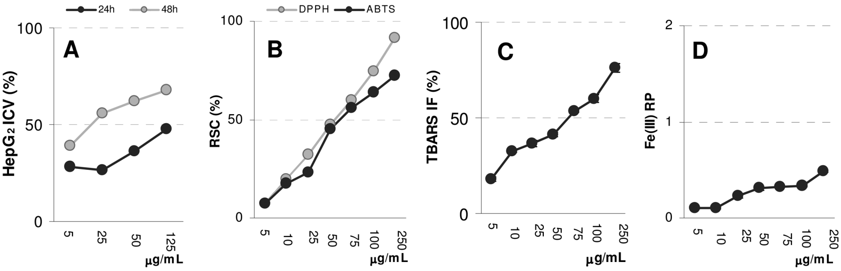
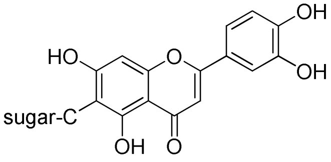


Figure 4. Bioactivity of P. velutina extract. A=HepG2 Cell Inhibition Viability; B=Radical Scavenging Capacity; C=TBARS Inhibition Formation; D=Fe(III) Reducing Power. Data are reported as percentage vs blank±SD (graphs A-C) or as increase of absorbance vs blank ±SD (graph D).

The plant extract was not able to induce the SOS DNA repair system and did not have the capability to decrease the activity of the known genotoxin 4-nitroquinoline and a slight increase of 4-nitroquinoline activity was observed starting from 20 µg/mL. No acute and chronic aquatic toxicity was found both for rotifers and crustaceans. The results of the extract examined with the YES-assay indicate a maximum capability to induce the expression of β-galactosidase of 70% at 5 µg/mL), in comparison to E_2 (100%) indicating that this extract is a partial estrogenic receptor agonist.

The metabolic profiling of the plant extract didn not allow the identification of specific metabolites. The 1D and 2D NMR data indicated the presence of flavones of luteolin bonded to sugar moieties throught a *C*-glycosidic bond with the C-6 carbon. The correlation regarding the B-ring of the flavones was in good accordance with a catechol ring.



Other signals in the upfield region of the spectra could agree with those of terpenoid structures, probably present as saponins, frequently reported from different Caryophyllaceae.

The antiproliferative activity against human hepatoblastoma cancer cell line HepG2 of pure C-glycoside flavons from *P. velutina* has been analyzed (*75*). Among them, isoorietin significantly reduces the cell proliferation after 48 h of exposure.

Rosmarinus officinalis L.

Rosmarinus officinalis (Lamiaceae) is a woody perennial evergreen plant with aromatic leaves and verticillasters few-flowered, in short axillary racems. There are just two species, *R. officinalis*, widespread in the mediterranean region, and *R. eriocalyx*, native to northwest Africa and southern Spain. The latter differs from the well-known rosemary in its smaller leaves, and densely hairy flower stems. It also tends to be lower-growing, often under 25 cm high and prostrate, and never exceeding 1 m high (*R. officinalis* can reach as high as 1.5 m, exceptionally 2 m). *Rosmarinus officinalis* is a steno-mediterranean plant naturally occurring in *macchia* vegetation mainly on calcareous soil (*34*, *35*). The fresh and dried leaves, very fragrant, are frequently used in traditional mediterranean cuisine; they have a bitter, astringent taste and are usually added as spices to a wide variety of foods. A tisane can also be obtained from them (*78*).

The phytochemical investigation of this species evidenced the main presence of abietane diterpenes, including several diterpenoid quinones (*79–81*), but triterpenoids (*82*) and flavonoids (*83*, *84*) have been also isolated. Recently two new abietane-type diterpenoid o-quinones, named rosmaquinone A and B, have been isolated from the aerial parts of *R. officinalis* (*85*). Other polyphenol present in *R. officinalis* is rosmarinic acid, widely distributed in the Lamiaceaeae herbs, which showed the highest concentrations of all the polyphenols in all organs of this plant (*78*).

The antioxidant properties of rosemary crude extract and its constituent compounds have been well studied. Most of these reported antioxidant activities based on cell-free biochemical tests of either crude extract or chemically fractionated constituents of rosemary. In this study, we employed different biochemical tests and a cell-based in *vitro* assay confirming rosemary antioxidant properties (Figure 5). The extract showed a remarkable antilipoperoxidative effect with an ID_{50} of 9.2 µg/mL.

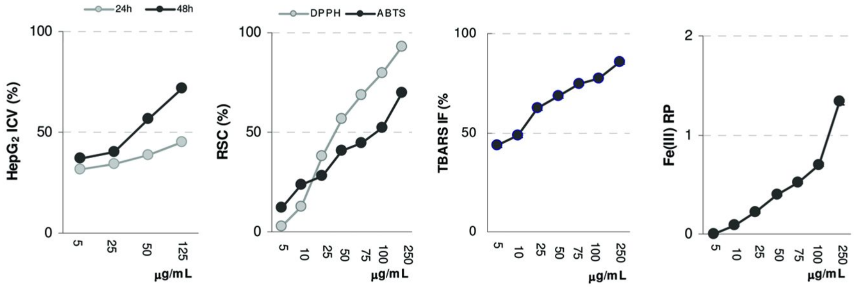
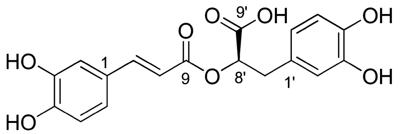


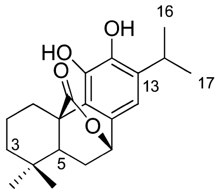
Figure 5. Bioactivity of R. officinalis extract. A=HepG2 Cell Inhibition Viability; B=Radical Scavenging Capacity; C=TBARS Inhibition Formation; D=Fe(III) Reducing Power. Data are reported as percentage vs blank±SD (graphs A-C) or as increase of absorbance vs blank ±SD (graph D).

The antioxidant properties of rosemary are probably responsible of the antigenotoxicity found for this extract which completely suppressed the 4-nitroquinoline genotoxicity. Obviously, no extract genotoxicity was found. The results of the aquatic toxicity were peculiar evidencing an acute lethal concentration (LC50) value of 74 μg/mL for the crustaceans and 1.19 μg/mL for the rotifers. These less evolved organisms of the aquatic chain showed a chronic toxic effect of rosemary at concentrations three order of magnitude less than the acute value. The estrogenic potential of rosemary extract was 56% and it was obtained at the lowest concentration when compared to the other extracts (0.31 μg/mL).

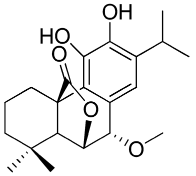
The ^{1}H NMR of the *R. officinalis* extract showed the presence of rosmarinic acid, a dimeric cinnamic acid. The H-8 and H-7 protons were evident in the ^{1}H NMR spectrum as two doublets at δ 6.29 and 7.49. The C-7 carbon at δ 148.7, showed, in the HMBC experiment, correlations with the H-2 and H-6 protons of the aromatic ring. Furthermore one proton of the H-7' diastereotopic methylene, which was evident as a doublet of doublet at δ 2.92, showed correlations with the C-2' and C-6' carbons of the second aromatic ring. In the upfield region of the spectrum, methyl signals, attributed to abietane diterpenes, were evident. In particular the H-16 and H-17 methyl signals of carnosol at δ 1.18 and 1.20 correlated, in the HMBC spectrum, with the C-13 carbon at δ 131.8. Whereas, the H-18 and H-19 methyls correlated with the C-3, C-4 and C-5 carbons, respectively at δ 42.1, 35.7, and 50.0. Similar correlations were evidenced for epirosmanol, but the H-18 and H-19 methyls at δ 1.17 and 1.19 showed heterocorrelations with the C-7, C-8 and C-9 carbons at δ 95.5, 32,2, and 53.6.



rosamarinic acid



carnosol



epirosmanol

Molecules as rosmarinic acid and carnosol are already widely known in literature for their important biological properties. Great deal of knowledge on the activity of carnosol is related to its ability to act as an antioxidant. It has been reported that the carnosol, together carnosic acid, are responsible for about 90% of the antioxidant activity performed from the rosemary leaf extract. Moreover, carnosol shows hepatoprotective effects and inhibits nitric oxide production in activated macrophages (*86*). The antiplatelet activity of the molecule has been also investigated: it has been shown that carnosol inhibited platelet aggregation by inhibition of TXA2 receptor and cytosolic calcium mobilization (*87*).

On the other hand, it has been reported that rosmarinic acid acts as adstringent, antioxidant, anti-inflammatory, antimutagen, antibacterial and antiviral (*88*). A theoretical calculation based on the density functional theory (DFT) has been performed to understand the antioxidant activity of rosmarinic acid (*89*). Furthermore, rosmarinic acid was widely studied for its antimicrobial and complement inhibition properties (*90*, *91*). Chemopreventive effects of rosmarinic acid were also demonstrated. The molecule is considered as a promising agent to prevent mesangioproliferative glomerular diseases. In fact, it is able to inhibit citokine-induced mesangial cell proliferation, to suppress PDGF (Platelet-Derived Growth Factor) and c-myc mRNA expression in PDGF-stimulated mesangial cells (*92*).

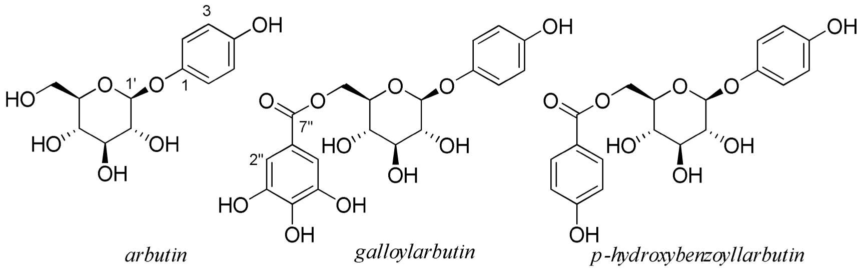
Arbutus unedo **L.**

Arbutus unedo (Ericaceae) is an evergreen shrub or small tree reaching as high as 5-10 meters, rarely 15 meters. *Arbutus* is a genus of at least 14 species of flowering plants native to warm temperate regions of the mediterranean area, western Europe, and North America. *Arbutus* species are characterized by red flaking bark and edible red berries. Fruit development is delayed for about five months after pollination, so that flowers appear while the previous year's fruits are ripening.

Arbutus unedo, also known as strawberry tree, is a steno-mediterranean species growing in limy soils of *macchia* vegetation, wood-margins and rocky slopes from 0 to 800 m a.s.l. It prefers well-drained soil and only moderate amounts of water (*34*, *35*). *Arbutus* species are used as food plants by some *Lepidoptera* species. Several species are widely cultivated as ornamental plants outside of their natural habitats. In Portugal the fruit is sometimes distilled into a potent brandy known as *medronho* (*93*).

A previous phytochemical study of *Arbutus unedo* reported a number of compounds, such as flavan, steroid and terpenoids (*94*). The leaves of strawberry tree were investigated to determine their flavonoid composition, and five flavonol glycosides were isolated and characterized: afzelin (kaempferol 3-rhamnoside), juglanin (kaempferol 3-arabinoside), avicularin (quercetin 3-arabinofuranoside), quercitrin (quercetin 3-rhamnoside), and hyperin (quercetin 3-galactoside) (*95*). Furthermore, some iridoid glycosides have also been isolated from the organic extract of the plant (*96*, *97*). Recently, from the hydroalcoholic extract of the strawberry tree, collected in a woodland dominated by *Arbutus unedo*, twelve hydrophilic compounds, ethyl gallate, arbutin, two arbutin derivatives, and eight flavonoids, have been identified (*98*).

Antioxidant colorimetric evaluation by DPPH and ABTS methods underlined the plant efficacy (fig. 6). The tested extract reduced massively DPPH radical. The addition of the extract dose of 25.0 μg/mL, after only ten minutes of reaction, is capable of establishing a visible conversion of the radical DPPH (purple) in its reduced form (yellow) estimable in a reduction of 61.9%. In accordance with antioxidant activity, a high total phenols content was found in methanol extract (87.7 mg GAE/g DM). The extract showed a peculiar antilipoperoxidative power with an ID_{50} of 0.3 μg/mL.



arbutin *galloylarbutin* *p-hydroxybenzoyllarbutin*

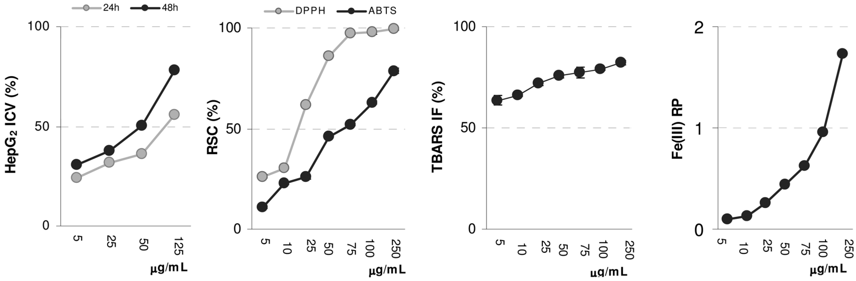


Figure 6. Bioactivity of A. unedo extract. A=HepG$_2$ Cell Inhibition Viability; B=Radical Scavenging Capacity; C=TBARS Inhibition Formation; D=Fe(III) Reducing Power. Data are reported as percentage vs blank±SD (graphs A-C) or as increase of absorbance vs blank ±SD (graph D).

No activation of SOS DNA repair system was detected for *A. unedo* extract while the capability of lowering the genotoxic activity of the standard was found starting from 100 µg/mL reaching 57% reduction at the maximum concentration tested. A weak acute toxicity in the order of dozen of µg/mL was found for crustaceans as well as for rotifers even if the EC50 for the long term exposure of these latter organisms was one order of magnitude lower. The extract was partial oestrogen receptor agonist with a RIE of 62% at 5 µg/mL.

The methanol extract of *A. unedo* is mainly constituted by arbutin. In the ^{1}H NMR spectrum the H-2/H-6 and H-3/H-5 aromatic protons were present as two doublets at δ 7.06 and 6.81, respectively. The observed correlations in the HMBC experiment confirmed the identity of the molecule. Among the other metabolites of this plant galloylarbutin was also detectable by the presence of a singlet at δ 7.10, due to the H-2 and H-6 omotopic protons of the acidic moiety, which correlated with the C-7", C-3"/C-5" and C-4" carbons at δ 168.0, 145.2, and 139.2, respectively. Furthermore, the presence of *p*-hydroxybenzoylarbutin was revealed by the presence of two doublets of the AA’BB’ system at δ 7.86 and 6.63. Several flavonols have been also detected by the presence of the H-6 and H-8 protons of the A-ring, identified as myricetin, quercetin, and kaempferol on the basis of the characteristic chemical shifts of the aromatic B-ring protons. The presence of a doublet at δ 0.98, of the anomeric protons at δ 4.38, 4.63, and 5.24, indicated their rhamnosyl, glucosyl and arabinosyl glycosides, already reported from this species.

Strawberry tree leaves had a long use in traditional medicine due to its antiseptic, diuretic, astringent and depurative properties (*99*, *100*). The main extract constituent, arbutin, is therapeutically used as an anti-infective for the urinary system as well as a diuretic. It is also an inhibitor of melanin formation and a skin-lightening agent that is included in compositions used for treating skin cancer. Recently, it has been reassessed the antioxidant activity of the molecule (*101*).

Myrtus communis L.

Myrtus communis (Myrtaceae) is an evergreen shrub or small tree that can reach as high as 5 meters. The leaf is entire, 3–5 cm long, with a fragrant essential oil. The fruit is a round blue-black berry containing several seeds. It is a steno-mediterranean species occurring on most arid and thermophilous aspect of macchia and woodland vegetation from 0 to 500 m a.s.l (*34*, *35*). The species is widely cultivated, since ancient times, as an ornamental shrub, for its abundants flowers blooming in later summer. Moreover it is used to produce an aromatic liqueur called "*mirto*" by macerating it in alcohol and comes in two varieties: "*Mirto Rosso*" (red *mirto*) produced by macerating the berries, and "*Mirto Bianco*" (white *mirto*) produced from the leaves (*102*).

Myrtus communis L. is one of the most important aromatic and medicinal species from the Myrtaceae family. Phytochemical investigations spanning several decades afforded various monoterpenoids, flavonoids (mainly galloyl-derivatives of catechin and gallocatechin) (*103*), and triterpenes (*104*). In the 1970s, Israeli scientists reported the isolation of a phloroglucinol antibiotic, named myrtucommulone A from myrtle leaves (*105*). Related compounds were subsequently reported by Appendino et al. (*106*). Leaves of *Myrtus communis* L. contain small amounts of phenolic acids (caffeic, ellagic and gallic acids) and quercetin derivatives (quercetin 3-*O*-galactoside and quercetin 3-*O*-rhamnoside), whereas catechin derivatives (epigallocatechin, epigallocatechin 3-*O*-gallate, epicatechin 3-*O*-gallate) and myricetin derivatives (myricetin 3-*O*-galactoside, myricetin 3-*O*-rhamnoside) are present in large amounts.

The bioassay results of methanolic extract of *M. communis* are reported in Figure 7. The extract was rich in phenols (86.3 mg GAE/g DM). ABTS and DPPH scavenging capacities showed relevant differences with ID_{50} values of 183.7 and 22.5 μg/mL, respectively (Figure 7B). The Fe(III) reducing power became significant when the highest dose was tested (Figure 7D). Myrtle extract decreased the HepG2 cell viability in a time-dependent manner (Figure 7A) showing, after 48 hours treatment, an ID_{50} of 34.6 μg/mL.

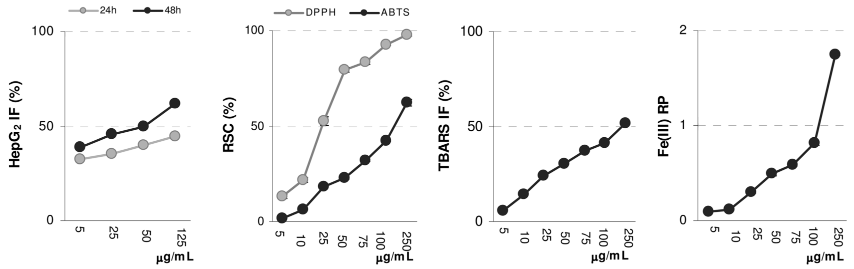
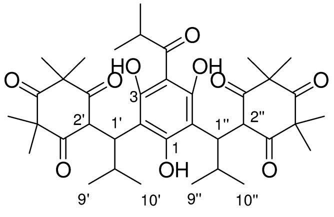


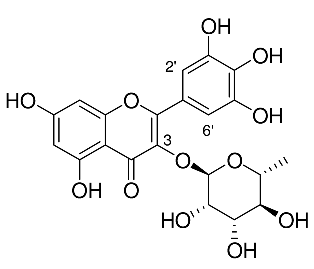
Figure 7. Bioactivity of M. communis extract. A=HepG$_2$ Cell Inhibition Viability; B=Radical Scavenging Capacity; C=TBARS Inhibition Formation; D=Fe(III) Reducing Power. Data are reported as percentage vs blank±SD (graphs A-C) or as increase of absorbance vs blank ±SD (graph D).

As well as the other extracts examined even *M. communis* did not show any genotoxicity at the SOS Chromotest. At the antigenotoxicity test any decrease of the standard genotoxicity was found. Both for rotifers and crustaceans the acute toxicity results were in the order of dozen of µg/mL. The chronic exposure of rotifers to this extract did not allow to determine an EC50 value until the concentration of 5 µg/mL. From the high amounts of catechin derivatives reported in the literature it would have been expected a positive response at the YES test.

The ^{1}H NMR of the plant extract showed the presence of several flavonoids, but, in this case the catechin signals were not detected, confirming the endocrine interference results. In particular, the myricetin substructure was revealed by the presence of characteristic singlet signal at δ 7.30 or 6.95. This latter signal is diagnostics for the 3-*O*-rhamnosylmyricetin. The presence of galloyl moiety was revealed by the singlets at δ 6.69 and 6.94, which correlated, in the HMBC experiment with the carboxyl carbon at δ 167.9.



myrtucommulone A



3-O-rhamnosylmyricetin

In the same experiment the presence of several carbonyl carbons, ranging from 200 to 220 ppm, correlated with shielded protons in the range between 1.00 and 1.30 ppm, suggested the presence of phloroglucinols. In particular the presence of myrtucommulone A was revealed by the observed correlations between the proton at δ 0.82 (H-9' and H-10') and the carbons C-8', and C-1' at δ 27.4, and 41.9, respectively.

The protective effects of flavonoids in biological systems has been widely reported ion the literature (*107*). The phloroglucinol myrtocummulone has been reported as responsible for the healthly efficacy of this plant, including the antibacterial activity (*108*), antioxidant properties (*109*), and in vivo anti-inflammatory effectiveness (*110*). Furthermore, it has been found that this metabolite induces apoptosis in cancer cell lines via the mitochondrial pathway involving caspase-9 (*111*).

Conclusions

Spices and herbs are recognized sources of natural antioxidants that can protect from oxidative stress and thus play an important role in the chemoprevention of diseases correlated to reactive radical species. It is known that plants accumulate antioxidant chemicals as secondary metabolites through evolution as natural means of surviving in a hostile environment. Considering the enormous biodiversity resources of the Mediterranean area, seven wild Mediterranean plants, some of them well known as medicinal plants, were screened for their total phenolic content, antioxidant capacity, genotoxic and antigenotoxic effects as well as antiproliferative activity on HepG2 cells. The evaluation of acute and chronic toxicity were also carried out. NMR-based metabolic profiling was assessed to identify the potential bioactive metabolites in the investigated plant extracts.

The enormous variability of the metabolites that constitute and characterize each investigated species seems to be mainly responsible for the exhibited antioxidant and antiproliferative effects.

The overall assessment can be shown through as in Table 2 where the analyzed biological properties of plants are summarized in terms of high, medium, low or no effect.

Shrub species, *Rosmarinus officinalis* and *Arbutus unedo*, show a considerable antigenotoxic potential which was probably due to the antioxidant activity of their components. In particular, *Rosmarinus officinalis* represents a clear source of antioxidant and phytoestrogenic compounds.

Comparative analysis of potential anti-tumor effects highlighted the marked activity of *Petrhoragia velutina* and *Carex distachya* root component, as well as of plants, such as rosemary, whose use is known in traditional medicine.

The data obtained by metabolomic and bioactivity screening of the investigated extracts suggest their use as promising sources of potential antioxidants. This hypothesis could assume its concreteness once the real health value and harmlessness of such matrices and the real pharmacological action of their components will be defined. Thus future studies will be aimed at the investigation of the effects on cellular mechanisms regulation and upon understanding the substances actually responsible for the antioxidant effects of the plant extracts.

Table 2. Biological effects of investigated Mediterranean plants

		Carex distachya		*Teucrium chamaedrys*		*Teucrium polium*		*Petrorhagia velutina*	*Rosmarinus officinalis*	*Arbutus unedo*	*Myrtus communis*
		L	R	L	R	L	R	L	L	L	L
Radical scavenging activity	DPPH		⬩	♦	♦	♦	♦	♦	♦	◆	◆
	ABTS	⬩	⬩	◆	◆	♦		♦	⬩	♦	⬩
Antilipoperoxidation	TBARS	⬩	⬩	♦	◆		⬩	♦	◆	◆	⬩
Reducing power	Fe (III)	⬩	♦	♦	♦	♦	♦		◆	♦	◆
Antioxidant	FCR	♦	♦	◆	◆	♦	♦	♦	♦	◆	◆
Antiproliferative	MTT (24 h)		⬩							⬩	
	MTT (48 h)	⬩	◆		♦		♦	◆	◆	◆	◆
Estrogenicity	YES-test	⬩		♦			⬩	♦	◆	♦	
Antigenotoxicity	SOS Chromotest	♦	⬩						◆	♦	
Genotoxicity	SOS Chromotest										
Chronic toxicity	*B. calyciflorus*	⬩	♦	♦	♦	♦	♦		◆	⬩	
Acute toxicity	*B. calyciflorus*		♦						◆	♦	♦
	T. platyurus		⬩						♦	♦	♦

◆ = *high*; ♦ = *medium*; ⬩ = *low*; lack of symbols indicates *no effect*.

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Chapter 13

Beyond Olive Oil: Active Components and Health Aspects of Some Less Studied Mediterranean Plant Products

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Current advances on health benefits of Mediterranean diet components considered foods or functional food items, namely legumes, aromatic plants, Chios mastic, and currants, provide significant guidance for further research and for industries in developing nutraceutical products. Cooked legume polyphenols (13.4-25.9 mg gallic acid eq (GAE) /100g) – mainly flavonoids in lentils and chickpeas, phenolic acids in the others – and triterpenic acids (0.29-8.55 mg/100g) inhibit LDL oxidation *in vitro*. The infusions of 12 aromatic plants (polyphenolic content between 5.3-159.2 mg GAE/cup) exhibit potent antiradical activity (7.7-201.3 mg Trolox® eq/cup) and ferric ion reducing antioxidant power (FRAP) values between 0.5-66.5 mg ascorbic acid eq/cup. Terpene rich Chios mastic exhibits potent antioxidant, anti-inflammatory and *in vivo* immunoregulating activity in inflammatory bowel disease. Polyphenol rich currants (151-246 mg GAE/100g – mainly phenolic acids) exhibit antiradical, LDL antioxidant and mononuclear cell cytoprotective activity. Further, currants induce death and exhibit antiproliferative and anti-inflammatory effect in gastric adenocarcinoma cells.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
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Introduction. World Mortality Pattern Changes

According to the 2008 WHO health report (http://www.who.int/whr/2008/whr08_en.pdf), there is a striking shift in distribution of death and disease from younger to older ages and from infectious, perinatal and maternal causes to noncommunicable diseases– including depression, diabetes, cardiovascular disease and cancers (*1*).

There is increasing evidence that oxidative stress is implicated in the pathogenesis of many inflammatory and degenerative diseases (*2*, *3*). Oxidative stress occurs when the oxidant-antioxidant balance becomes too favourable to prooxidants. In modern Western medicine, the balance between antioxidation and oxidation is believed to be a critical concept maintaining a homeostasis (*4*, *5*).

Current hypotheses favour the idea that regulating oxidative stress can provide clinical benefits. As dietary habits are associated with several chronic diseases -including cardiovascular disease, cancer and immune dysfunction- the nutritional approach could be a useful tool for lowering oxidative stress. Indeed, there are now well established nutritional recommendations for prevention of cancer, atherosclerosis and other chronic diseases.

The traditional Mediterranean diet of Greece is considered beneficial against chronic and degenerative diseases (*6*). It is characterized by reduced intake of saturated lipids and animal proteins, and high intake of olive oil, vegetables, fruits, grains, potatoes, nuts, legumes which provide significant amounts of phytochemicals.

Phytochemicals are bioactive plant components, which do not provide energy, usually exhibit antioxidant action, and exert beneficial effects for human health (cardioprotective, anti-inflammatory, anti-tumour etc). Examples of phytochemicals are tocopherols, polyphenols, terpenes, carotenoids, ascorbic acid etc.

Although the most characteristic feature of Mediterranean type diets is the consumption of high amounts of olive oil, other less known Mediterranean plant products exhibit potential health effects due to their significant content in bioactive microconstituents and obviously contribute to the overall beneficial effects of such diets.

This chapter presents the main phytochemicals and the antioxidant capacity of some less studied Mediterranean plant foods, in the form they are actually consumed, and establishes their potential health benefits, *in vitro* and/or *in vivo*. The plant foods selected for this study are (a) cooked dry legumes, (b) infusions of aromatic-medicinal plants, (c) *Pistachia Lentiscus* resin (Chios mastic gum), (d) currants and sultanas (dried vine fruits).

Legumes

Legumes have long been associated with longevity food cultures. For example, the Japanese eat soy, tofu, natto and miso, the Swedes eat brown beans and peas and the Mediterraneans eat lentils, chickpeas and white beans (*7*–*9*). Legumes contain almost twice the amount of protein compared to cereal grains, while they are low in fat, and rich in complex hydrocarbons and minerals (*10*).

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Furthermore, legumes exhibit lower glycaemic indices compared to other starchy foods and contain phytosterols, natural antioxidants and bioactive carbohydrates, which are considered potentially beneficial for human health (*11–13*).

Both epidemiological and clinical intervention studies showed that legume consumption is inversely associated with the risk of coronary heart disease, obesity, and type II diabetes mellitus (*14–17*), while it results in lower LDL and higher HDL cholesterol levels (*18*, *19*). Nowadays legumes gain increasing attention as functional food items and increase in their consumption within the Western diet is recommended (*20*, *21*). It should be noted hereby that legumes contain certain antinutritional factors, among them trypsin inhibitors, α-galactosides, and phytic acid (*22–24*), which are normally diminished during legume cooking.

With the exception of soybean, there is relatively little information on the phytochemical content of cooked legumes (*25*). To investigate the phytochemical content of dry cooked legumes Kalogeropoulos et al (*26*) prepared several kinds of legumes that are often consumed in Greece and other Mediterranean countries, by fisrt soaking and then boiling them in tap water. The obtained cooked legumes were subsequently homogenised, freeze-dried and analysed for phytosterols, tocopherols, simple polyphenols and triterpenic acids (*26*). In addition, in the same cooked legumes total phenolics were determined; DPPH$^\bullet$ radical scavenging activity and ferric ion reducing antioxidant power (FRAP) were also assessed. Finally, the potential of the cooked legumes to inhibit human LDL oxidation *in vitro* was tested. The results obtained for seven kinds of legumes, namely broad beans, chickpeas, yellow split peas, small lentils and 3 varieties of white beans (medium sized, giant, and elephant beans) are presented and discussed.

Phytochemicals in Cooked Legumes

Tocopherols and Phytosterols

Tocopherols are considered as very effective lipid phase antioxidants, acting as peroxyl radical scavengers that terminate chain reactions in membranes and lipoprotein particles (*27*). Kalogeropoulos et al (*26*) reported tocopherols – mainly β- and γ- homologues – to be present at concentrations ranging from 0.26 - 0.36 mg/100g in white beans and broad beans to 0.82 and 1.78 mg/100g in lentils and chickpeas, respectively (*26*), their values being one order of magnitude lower than the respective reported by other researchers for uncooked legumes (*11*, *28*), due to soaking and boiling.

Phytosterols were also present in the cooked legumes at concentrations that ranged from 22.9 – 48.9 mg/100g in lentils and chickpeas, respectively (*26*), *with* β-sitosterol predominating in all cases. As a result of soaking/cooking the phytosterols present in the cooked legumes were 4-8 times lower than the values reported for uncooked legumes (*28*), still representing the 15.8-24.4% of phytosterols daily dietary intake (*26*).

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Simple Polyphenols

Polyphenols are known to exert antioxidant anti-inflammatory and antimicrobial action, while accumulating literature data indicate that they protect body tissues against oxidative stress; they have been shown to be protective *in vitro* against several types of cancer, such as breast, prostate, skin, and colon cancer (*29*). There is now emerging evidence that their metabolites exert modulatory effects in cells through selective actions on different components of the intracellular signalling cascades, vital for cellular functions such as growth, proliferation and apoptosis (*25*). There are several reports on the presence of polyphenols in uncooked legumes, but relatively fewer studies on the respective content of cooked or processed legumes, as reviewed recently by Amarowizc and Pegg (*11*). Phenolic acids and flavonoids are the commonly reported simple phenolics in legumes (*11*, *13*, *30–34*).

Kalogeropoulos et al. (*26*) reported the presence of both phenolic acids and flavonoids in several kinds of cooked legumes. Among the phenolic acids caffeic acid, cinnamic acid, *o*-coumaric acid, *p*-coumaric acid, gallic acid, ferulic acid, *p*-hydroxybenzoic acid, phloretic acid, protocatechuic acid, sinapic acid, and vanillic acid were present in the majority of cooked legumes (*26*) studied. Among the flavonoids determined in cooked legumes, catechin and epicatechin predominated followed by chrysin, genistein, quercetin and kaempferol (*26*). The same flavonoids together with apigenin, luteolin, daidzein and coumestrol are present in raw, cooked or germinated leguminous seeds and their extracts, according to a recent review by Amarowicz and Pegg (*11*). Lentils have been reported to be consistently rich in flavonoids in raw (*35*) or cooked form (*26*).

Triterpenic Acids

Triterpenic compounds are common constituents of plants. They are relatively non-toxic and possess pharmacological properties exerting anti-inflammatory, hepatoprotective, antitumour, antiviral, anti-HIV, antimicrobial, antifungal, antidiabetic, gastroprotective and antihyperlipidemic action. The triterpenic acids oleanolic, ursolic and maslinic were present in the seven cooked legumes studied in concentrations ranging from 0.34-8.55 mg/100g in medium beans and chick peas, respectively. Oleanolic acid was present in all cooked samples; ursolic acid was present in broad beans, chick peas and yellow split peas, while chick peas and lentils contained in addition maslinic acid.

Total Phenolic Content and Antioxidant Activities of Cooked Legumes

The total phenolic content, together with the DPPH$^{\bullet}$ free radical scavenging capacity and the FRAP values of the cooked legumes studied are presented in Figure 1. The total phenolic content of the legumes studied ranged from 13.4 mg of gallic acid equivalents (GAE) per 100g in medium beans to 25.9 mg GAE/100g in small lentils. These values are generally lower than those reported for uncooked legumes. The obvious explanation for this decrement is the soaking and boiling of legumes, which results in partial leaching and thermal/oxidative destruction of phenolics, in agreement with previous reports on the effect of soaking and cooking on total phenolic contend of several types of legumes (*33*, *36–39*).

The DPPH scavenging activity of cooked legumes ranged from 0.24 mmol Trolox equivalents (TE) /100g in broad beans to 2.1 mmol TE/100g in lentils, while the FRAP values ranged from 1.3 μmol ascorbic acid equivalents (AAE) /100g in elephant beans to 7.1 μmol AAE/100g in lentils (Figure 1), values that are lower than those reported by Xu and Chang (*40*) for uncooked peas, chickpeas, lentils, kidney beans and black beans.

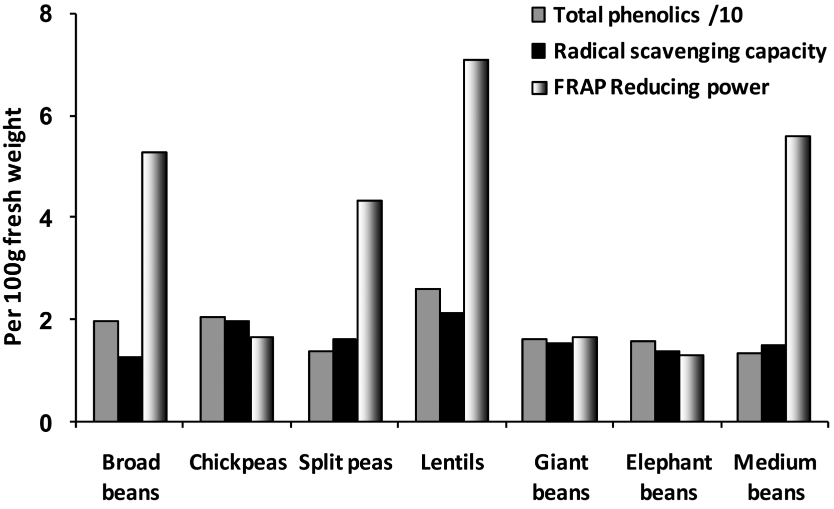

Figure 1. Total phenolic content (mg GAE, divided by 10), free radical scavenging activity (mmol TE) and FRAP reducing potential (μmol AAE) of 100 g cooked legumes; GAE stands for gallic acid equivalents;TE stands for Trolox® equivalents; AAE stands for ascorbic acid equivalents.

In Vitro Inhibition of LDL Oxidation by Cooked Legumes

Human low density lipoprotein (LDL) particle is recognized as being highly vulnerable to oxidation by reactive oxygen species (ROS), leading to the formation of oxidized LDL (oxLDL) which is strongly implicated in atherogenesis (*41*, *42*).

The potential of cooked legumes to inhibit the Cu^{2+} induced LDL oxidation, was tested *in vitro*. For this purpose, LDL oxidation in the presence or absence of cooked legumes' extracts was followed, and the respective lag phase times were compared.

Amounts of LDL and legume extracts used were calculated to represent the LDL in an adult's circulation –with blood volume of 4L and LDL concentration equal to 150 mg/dL (*43*) –and the extract obtained from one exchange (0.5 cup = 115 g) of cooked legumes, respectively. As shown in Figure 2, all samples tested exhibited inhibitory activity against LDL oxidation with the relative effectiveness

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increasing in the order: split peas < broad beans < giant beans < elephant beans < chick peas < medium beans < lentils. Lentils have been reported to exhibit higher inhibitory activity against LDL oxidation compared to other food legumes (*40*). The inhibitory ability of the legumes studied, correlated better with flavonoids and free radical scavenging capacity, while it was not correlated with phytosterols, squalene, tocopherols or terpenic acids content (data not shown). Flavonoids have been found to exert anti-inflammatory activity (*44*) and to act against LDL oxidation, their antioxidant capacity being related to their chemical structures (*45*).

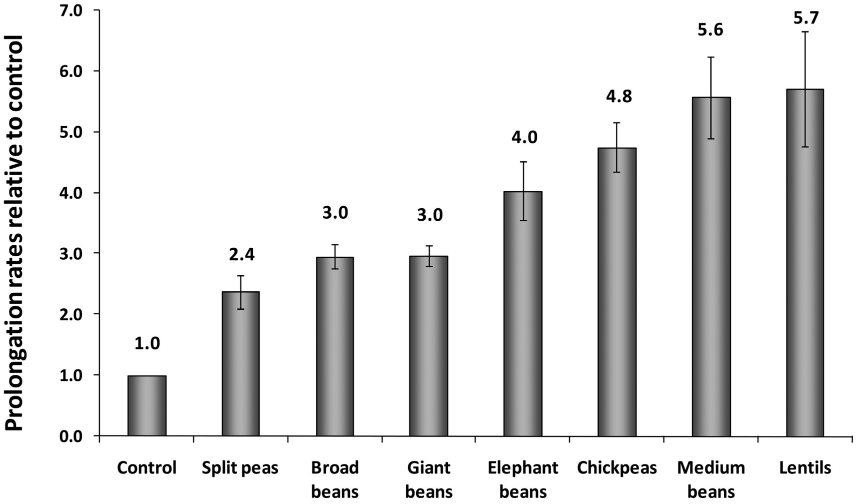

Figure 2. Antioxidant activities of cooked legume extracts on Cu^{2+}- induced LDL oxidation based on detection of conjugated dienes. The values represent the prolongation rates relative to control, from assays performed in duplicate. The amounts of LDL and legume extracts were calculated to represent the LDL in an adult's circulation and one cup (115g) of cooked legumes, respectively.

Infusions of Mediterranean Aromatic and Medicinal Plants

Herbal infusions are considered among the significant sources of dietary polyphenols (*46*), exerting antioxidant activity which is mainly attributed to the presence of essential oils and polar phenolic compounds. Due to geomorphological characteristics, the flora of the Mediterranean basin presents high biodiversity with many endemic plants. The aromatic and medicinal plants of the Mediterranean have a long tradition of use as folk remedies and culinary herbs since antiquity. In Greece, herbs are consumed in the form of infusions prepared by one herb alone or –as in the case of St John's wort- in mixture with other aromatic plants.

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The composition and antioxidant activity of essential oils and extracts of aromatic herbs obtained by different solvents have been extensively studied. On the contrary, little is known about the presence of antioxidants in herbs' infusions and decoctions, which we actually consume (*47*, *48*). For this reason infusions of 12 aromatic plants, purchased from the island of Crete - except St John's wort which was purchased from Central Macedonia, Northern Greece, were prepared by boiling 3 g of dried aerial parts of the plants for 3-4 minutes in 200 mL (one cup) tap water. The plants selected (Table 1) are members of the Lamiaceae family, with the exception of *Hypericum perforatum* –Cluciaceae- and *Matricaria chamomilla* –Asteraceae. The infusions obtained were filtered, freeze-dried and analyzed for total phenolics, DPPH• radical scavenging activity and FRAP. In addition, simple polyphenols and terpenic acids were determined by GC/MS as previously reported (*49*).

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Table 1. Common and scientific names, and distribution of the selected aromatic plants

Common name	*Scientific Name*	*Distribution*
Cretan marjoram	*Origanum microphyllum*	Endemic, Crete island
Rosemary	*Rosmarinus officinalis*	Native, Mediterranean
Cretan dittany, dictamnus	*Origanum dictamnus*	Endemic, Crete island
Pink savory	*Satureja thymbra*	Native, Mediterranean
Thyme	*Thymus vulgaris*	Southern Europe
Marjoram	*Origanum Majorana*	Indigenous, Mediterranean
Oregano	*Origanum vulgare*	SW Eurasia, Mediterranean
Greek mountain tea	*Sideritis syriaca*	Endemic, Greece
St John's wort	*Hypericum perforatum*	Worldwide
Sage	*Salvia officinalis*	Native, Mediterranean
Pennyroyal	*Mentha pulegium*	Europe
Chamomile	*Matricaria chamomilla*	Europe

All the infusions contained significant amounts of phenolics, and exhibited free radical scavenging activity and reducing potential against Fe^{3+} (Table 2). Total phenolic content ranged from 5.3-159.2 mg GAE per cup (=200 mL), comparable to the values of 88 - 184 mg GAE per cup reported for Greek herbal infusions (*48*). DPPH• radical scavenging capacity ranged from 0.03-1.72 mmol TE per cup, and FRAP ranged between 0.5-66.5 mg AAE per cup (Table 2). In all cases the lower values were observed for the infusion of rosemary (*R. officinalis*) and the higher for that obtained from St John's wort (*H. perforatum*).

Table 2. Total phenolic content, free radical scavenging activity and reducing potential of the aromatic plants' infusions

Aromatic plant	*Total phenolics (mg GAE/cup*)*	*DPPH• scavenging activity (mmol TE/cup)*	*Ferric reducing antioxidant power, FRAP, (mg AAE/cup)*
O. microphyllum	17.1±2.1	0.21±0.01	4.7±0.17
R. officinalis	5.3±4.2	0.03±0.02	0.5±0.07
O. dictamnus	29.7±5.2	0.57±0.02	16.5±0.72
S. thymbra	52.6±1.3	0.52±0.01	17.0±1.4
T. vulgaris	32.5±5.5	0.28±0.02	6.8±0.15
O. majorana	59.2±1.3	0.60±0.01	19.1±1.1
O. vulgare	61.9±4.8	0.80±0.02	29.1±2.7
S. syriaca	21.5±3.8	0.21±0.02	6.7±0.08
H. perforatum	159.2±11.2	1.72±0.07	66.5±5.0
S. officinalis	18.7±1.8	0.21±0.01	4.7±0.41
M. pulegium	61.9±2.0	0.72±0.01	20.2±0.75
M. chamomilla	17.7±2.5	0.32±0.01	7.5±0.39

Results are means ± SD (n=3); * 1cup = 200 mL; GAE stands for gallic acid equivalents; TE stands for Trolox® equivalents; AAE stands for ascorbic acid equivalents.

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Simple polyphenols content, quantified by GC/MS, ranged from 0.14-38.0 mg/cup in *R. officinalis* and *H. perforatum*, respectively, and were mainly phenolic acids and flavonoids; Among 18 phenolic acids tested, 13 were present in more than 5 infusions, namely *p*-hydroxybenzoic acid, caffeic acid, cinnamic acid, chlorogenic acid, *o*-coumaric acid, *p*-coumaric acid, ferulic acid, gallic acid, 3,4-dihydroxy-phenylacetic acid, protocatechuic acid, syringic acid, sinapic acid, and vanillic acid. Total phenolic acids concentrations ranged between 0.12-5.96 mg/cup in *R. officinalis* and *M. chamomilla*, respectively (Figure 3).

Regarding flavonoids, the aglycons of epicatechin and catechin were present in all infusions, while aglycons of chrysin, genistein, kaempferol, naringenin, and quercetin were present in the majority of them, their summed concentrations ranging from 0.013-36.1 mg/cup in *R. officinalis* and *H. perforatum*, respectively (Figure 3). Additionally, the triterpenic acids oleanolic and ursolic were detected in the infusions of *O. microphyllum, R. officinalis, O. majorana, O. vulgare,*

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S. officinalis, and *M. pulegium,* at concentrations ranging between 0.014-0.11 mg/cup. The results obtained indicate that the consumption of one cup of the infusions studied provide certain amounts of polyphenols and terpenic acids, exhibiting significant antioxidant and radical scavenging activities.

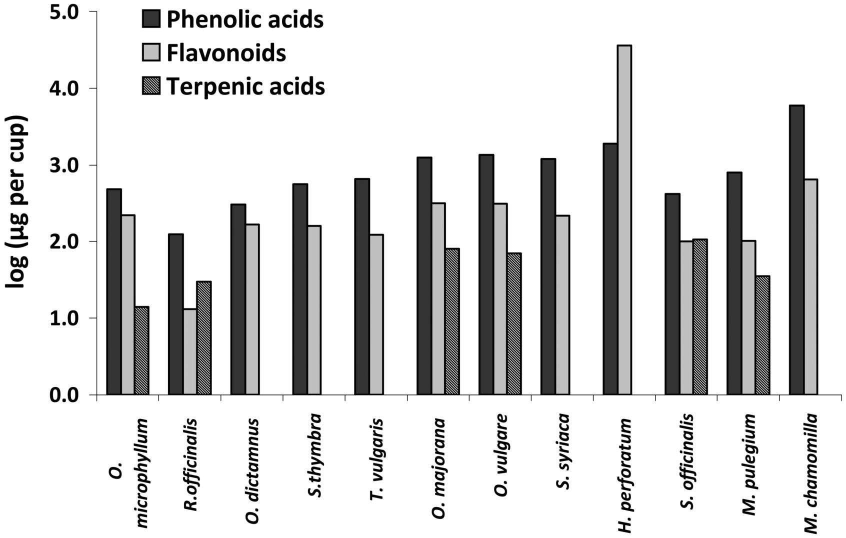

Figure 3. Phenolic acids, flavonoids and terpenic acids present in one cup (200 mL) of the aromatic plants infusions. Note that the scale is logarithmic.

Chios Mastic: A Tear That Pleases, Perfumes, Relieves, and Heals

Chios Mastic or "mastiha" is the name of a resinous sap produced from the mastic tree (*Pistacia Lentiscus* var. Chia). It is a natural, aromatic resin in teardrop shape, falling on the ground in drops from superficial scratches induced by cultivators on the tree's trunk and main branches with sharp tools.

As it drips, this sap appears as a sticky and translucent liquid, which 15-20 days later is solidified into irregular shapes influenced by the area's weather conditions in summertime that is intense drought and sunlight. After being solidified, it has a crystal form, while its rather bitter taste quickly subsides to leave a distinctive aroma that really makes it unique. That solid product is then harvested and washed by mastic growers, finally providing the natural Chios Mastic. Its colour is initially ivory-like but as time goes by that shade is lost and 12 to 18 months later it changes into yellowish due to oxidation. It is made of hundreds of components, and such multitude probably justifies the multiple uses of Chios Mastic, in the fields of food industry, health and cosmetic care, worldwide.

Chios Mastic has been recognized since ancient times both for its distinctive aroma and its healing properties. Many ancient Greek authors, including Dioscurides and Theophrastus, mentioned Chios Mastic for its healing properties in intestines, stomach and liver. It has been recorded as the first natural chewing gum in the ancient world. Since 1997, Chios Mastic has been characterized as a Product of Protected Designation of Origin (PDO), on the basis of Regulation No. 123/1997 (L0224/24-1-97) of the European Union and it has been registered on the relevant Community List of PDO Products. According to the above regulation, Chios Mastic is protected from the sale of any competitive imitation product whatsoever that would undermine the reputation of the Designation of Origin (http://www.gummastic.gr).

The lentisk is a rather resilient plant with minor demands that is why it grows well on arid, rocky and poor soil. As its roots are spread on the soil's surface, it can survive in conditions of absolute drought, but can be extremely sensitive to cold and frost. New cultivations are produced from old trees' branches (grafts) and the old ones are renewed from offshoots or layers.

Chios Island is actually identified with mastic and while there are lentisks all over the island, mastic is only produced in the southern part of Chios, in the so-called Mastihohoria or mastic villages, where the climate is especially warm and dry. Besides a longtime tradition, this "uniqueness" is probably due, to certain soil and weather conditions which favor the mastic tree's cultivation only in Chios and only in this specific part of the isle.

The medicinal and pharmaceutical properties of Chios Mastic were well known ever since antiquity. Nowadays, laboratory research and clinical studies have revealed that natural Chios Mastic is gifted with unique beneficial and therapeutic properties, thus confirming what has been historically recorded over the past.

The treatment of various gastric malfunctions with Chios Mastic has been documented. Earlier studies showed that mastic gum possesses anti-ulcer activities (*50–52*). Although the effects on *Helicobacter pylori* are controversial (*53–56*), a recent study showed that mastic administration (0.75 mg/day) reduces *H. pylori* colonization in stomach of infected mice ($p \leq 0.01$) (*57*). In 2002, the hepatoprotective effect of the aqueous extract from the leaves of *P. lentiscus* tree on CCl_4 intoxicated rats was published (*58*). Furthermore, the plant has been shown to suppress the extent of iron-induced lipid peroxidation in rat liver homogenates (*59*).

Chios Mastic, exported from Chios to all over the world, is the basis for the production of a great variety of mastic products, such as bakery products, sweets, jams, ice-creams, chocolates, chewing gums, candies, beverages, tea, coffee, dairy products, pasta, sauces, liquors, ouzo and wine. In certain areas of Greece, mostly of the Aegean Sea, mastic is often used as a flavouring for Easter sweets and for a delicious ice-cream known as kaimaki, which has an unusual chewy and stringy texture thanks to the addition of Chios Mastic as a thickening agent. Modern Greek chefs have proved that this spice with its unique aromatic, wood- and pine-like, exotic taste can go along with almost everything, from tomatoes in a tasteful sauce to white wine and lemon in most delicate sauces, and even to chocolate with which it makes a perfect match. In Lebanon and Syria they make

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a sort of traditional mastic-flavoured cheese. For Arabs, mastic is considered as a great luxury for flavouring food, sweets or milk. As a spoon sweet, mastic is served in a particularly traditional way, inside a glass of water, a version known as “ypovryhio” (submarine).

It is also used as an ingredient for ointments against burns. Rosin is a derivative of mastic used for the production of surgical stitches, while mastic oil is widely used in perfume and cosmetics industry.

Finally, thanks to its quality as a color stabilizer, mastic is used for the production of high grade varnishes.

Potential Antiatherogenic Effect of Chios Mastic

Effect on LDL Oxidation in Vitro

The evidence supporting the hypothesis that LDL is the major atherogenic lipoprotein comes from epidemiological studies, clinical trials, studies in laboratory animals, heritable hypercholesterolemias, pathologic investigations, and studies in model systems. The role of oxidized LDL (oxLDL) in atherosclerosis, and consequently in CVD, is manifold. oxLDL induces cell adhesion molecule expression in aortic endothelial cells (*60*). Enhanced endothelial cell expression of chemotactic and adhesion molecules (i.e. E-selectin), ICAM-1 (CD54), and VCAM-1 (CD106), within the artery wall result in monocyte binding to endothelial cells, their entry into the vascular system, their differentiation into macrophages, and final conversion into foamy cells. oxLDLs also stimulate T-cells through the major histocompatability complex (MHC) and CD4+ helper T-cell receptor (*61*). Stimulated T-cells secrete: (i) IL-1 that increases smooth muscle cell proliferation, (ii) IL-2 that activates monocytes and increases T-cell proliferation, and (iii) IFN-γ that induces MHC expression in endothelial and smooth muscle cells. Activated macrophages also secrete a number of cytokines (TNF-α, TGF-β, M-CSF, G-CSF, PDGF) that influence the expression of mediators of endothelial activation, such as NO. Besides, the aldehydic products of LDL oxidation exert a direct toxic effect on the endothelium and platelets.

To evaluate the potential of Chios Mastic to inhibit LDL oxidation *in vitro*, the method of Cu^{2+} induced oxidation was applied, using different volumes of Chios Mastic extracts, resuspended in ethanol (*62*).

The effect of the polar extract from 2.5mg of Chios Mastic was almost equal to that of 5mg, while 50mg was shown to be the quantity required for the complete inhibition of LDL oxidation. For comparative purposes, a protection factor, expressed as % protection factor, is used. When fractionating to determine a structure-activity relationship, mastic oil, collofonium residue and both the acidic fractions of NaOH and Na_2CO_3 were found potent inhibitors of LDL oxidation. Acidic and neutral fractions were comparatively inactive (8.9±9.2% and 19.6±18.7% protection, respectively) Normal collection obtained by simply cutting the tree, exhibited remarkable protective activity against LDL oxidation (75.3±19.5% protection) while ‘liquid collection’ obtained by injecting phytohormones to the tree appeared to have considerably reduced activity

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(39.8±21.0% protection), indicating that the chemical constituents of Pistacia lentiscus from 'normal' and 'liquid' type collection differ.

Furthermore, the pure triterpenes amyrin and oleanolic acid, which are natural constituents of Chios Mastic, exhibited relatively high protective activity (62.6±18.2% and 67.9±19.0% protection), however it did not exceed the overall activity of the crude product suggesting a synergistic effect between the active compounds. The isomers oleanolic and ursolic acids exhibited almost the same activity (67.9±19.0% vs 70.7±18.6 protection, respectively) indicating that structural differences do not influence the biological activity.

Effect on Peripheral Blood Mononuclear Cell (PBMC) Viability in Vitro

During the early atherosclerotic process, monocytes differentiate into macrophages that themselves effect modifications in LDL, most importantly taking up the endothelial cell modified LDL. The uptake of oxLDL occurs via scavenger receptors of the class SR-A and SR-B, a member of which is CD36 scavenger receptor that binds to its lipid moiety (*41*). Macrophages become susceptible to apoptosis. Nucleus shrinks; organelles change; membrane loses integrity; DNA breaks down. Eventually macrophages are converted to foam cells, full of cholesterol and oxidized lipids. Macrophage foam cells form the early atherosclerotic lesions documented as the pathogenesis of cardiovascular heart disease (*41*). Also, cell death of T cells is abundant in human atherosclerotic lesions having consequences on the evolution of the atheroma.

In an attempt to deepen our knowledge of the potential antiatherogenic and consequently cardioprotective effect of Chios Mastic, we employed a cell culture model of atherogenesis which included PBMC isolated from healthy volunteers and oxLDL (*63*). Oxidized LDL was found to be highly cytotoxic, resulting in severe damage of cultured cells, either apoptosis or necrosis, depending on the duration of exposure. The oxLDL toxicity on PBMC was almost minimized in the presence of Chios Mastic polar extract.

As far as monocytes are concerned, loading with oxLDL significantly increased CD36 and upregulated CD36 mRNA expression. The significance of enhanced CD36 expression is due to the fact that once macrophages attract oxLDL to scavenger receptors they promote endocytosis and convert into cholesterol-loaded foam cells. Nutrients can influence gene expression directly or via gene promoters, via control of regulatory signals in nontranslated regions, and via post-transcriptional pathways. All the genes encoding proteins and the genes associated with transcriptional activation and signals modulating the transcription of the respective genes are potential candidates for gene–diet interaction. The capacity of dietary antioxidants to modulate gene expression has been investigated chiefly during the last decade. The antiatherogenic effect of Chios Mastic extract was evident attributed to the downregulation of CD36 both at the protein and at the transcriptional levels, to inhibition of both apoptosis and necrosis and restoration of GSH levels.

The decrease of GSH (20.0%) in PBMC treated with oxLDL for 48h was similar to decrease (21.4%) in cells treated with the oxidizing agent for 72 h. The

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extract from Chios Mastic restored GSH levels utterly when treating cells under oxidative stress. Measurement of GSH levels after PBMC treatment individually with the polyphenolic and triterpenoid fractions revealed an increase 5.0% in GSH when treating cells with oxLDL and the polyphenolic fraction and 23.6% when treating cells with oxLDL and the triterpenoid fraction, compared to GSH measured when culturing cells with oxLDL alone. Consequently, triterpenes were the most active on the antioxidant defense of PBMC. Mass spectra has indicated oleanolic acid and urs-12-en-28-al as the major components of the triterpenoid fraction while indicated in the polyphenolic fraction were tyrosol, *p*-hydroxy-benzoic acid, *p*-hydroxy-phenylacetic acid, vanillic acid and traces of gallic and *trans*-cinnamic acids (*64*). Oleanolic acid and isomer ursolic acid have been found to increase glutathione and superoxide dismutase in Dahl salt-sensitive insulin resistant rat model of genetic hypertension (*65*). They have been also proven to exhibit antioxidant effect (*62*), antihyperlipidemic and antihypertensive effects and to prevent the development of atherosclerosis (*66*). Regarding polyphenols, these have been proven to be potent antioxidants (*67*).

Further to the above, when evaluating the cardioprotective effect of Chios Mastic in humans, subjects ingesting high-dose Chios mastic powder exhibited a decrease in serum total cholesterol, LDL, total cholesterol/HDL ratio, lipoprotein (a), apolipoprotein A-1, apolipoprotein B (apoB/apoA-1 ratio did not change), SGOT, SGPT and gamma-GT levels. In subjects ingesting low-dose Chios mastic powder glucose levels decreased in males (*68*).

The binding and recruitment of circulating monocytes to vascular endothelial cells are early steps in the development of inflammation and atherosclerosis, mediated through cell adhesion molecules that are expressed on the surface of endothelial cells. When evaluating the potential of the neutral extract of Chios Mastic to influence the expression of adhesion molecules and the attachment of monocytes in human aortic endothelial cells, inhibition of both the expression and attachment were observed, providing new insight into the beneficial effect of Chios Mastic on endothelial function (*69*).

Immunomodulation using Chios Mastic. The Case of Inflammatory Bowel Disease (IBD)

Regulation of the immune system includes a complex system of immune cells, pro-inflammatory and anti-inflammatory polypeptides and adhesion molecules.

The Inflammatory Bowel Disease (IBD) refers to ulcerative colitis and Crohn's disease. Incidence rates of 3 to 14 cases per 100,000 people are reported in the Western world (*70*), but pathogenesis remains unknown; genetic, environmental and immunologic factors seem to be responsible for IBD suffering, resulting in dysregulation of the immune system (*71*). Cytokines produced by immune cells contribute to the inflammatory response. Failure of controlling leukocyte recruitment enhances immune cell activation, which leads to further chemotaxis. Several inflammatory cytokines have been implicated in IBD and are elevated in colonic tissue and peripheral blood of IBD patients. TNF-α has been proven critical for IBD, but apart from TNF-α other cytokines play central roles in IBD. The proinflammatory cytokines IL-6 and IL-8 are produced in excess, while the immunosuppressive IL-10 is reduced in inflammed tissues. Both the excess of proinflammatory cytokines and the relative inefficiency of counterregulatory molecules are required for maintaining, amplifying, and perpetuating chronic inflammation in IBD (*72*). In addition, different cytokines induce the expression of adhesion molecules such as ICAM-1 on the endothelium, thus favouring the recruitment of new inflammatory cells. Imbalance between oxidant and anti-oxidant factors is also observed. In presence of inflammation reactive oxygen species inhibit antioxidant actions increasing lipid peroxidation. As a result, oxidative stress occurs. Corticosteroids, antibiotics and immunosuppressants are used to standardize symptoms (*71*).

With increasing awareness of the harm of the long-term use of corticosteroids the use of natural compounds in treatment of autoimmune disorders attracts wider research and seems to be embraced by patients. When patients with Crohn's disease were treated with Chios Mastic significant reduction in Disease Activity Index was reported (*73*, *74*). Effectiveness was probably owed to significant decrease of the pro-inflammatory IL-6 (Table 3), inducing remission in seven out of ten patients. The importance of IL-6 in patients with Crohn's disease has been well documented and mRNA for IL-6 is overexpressed in the inflamed mucosa of patients with active disease (*72*).

Elevated IL-6 in plasma of patients with Crohn's disease has been previously described (*75*). Because IL-6 is the main cytokine factor responsible for hepatic induction of acute phase proteins in Crohn's disease, significant decrement in CRP was reasonable (Table 3). Oxidative stress has been proven to upregulate IL-6 gene expression. Chios Mastic treatment resulted in increase of plasma Total Antioxidant Potential (TAP) in Crohn's disease patients (Table 3). Whether the antioxidant triterpenes and phenolics contained in Mastic act after being absorbed or act on the exposed gastrointestinal mucosa, remains uncertain. Although not as well absorbed as vitamins C and E, yet some phenolic compounds are absorbed, while those unabsorbed may remain in the lumen and become available for fermentation in the gut. Thus, gastrointestinal mucosa can be exposed to these compounds, or to their bacterial and systemic metabolites. Also, some triterpenes like glycyrrhetinic acid, the triterpene derivative of glycyrrhizin, have been shown to be bioactive in experimental gastric lesions.

The anti-TNF-α treatment in TNF-mediated diseases, such as Crohn's disease is developing. In active patients subjected to Chios Mastic treatment, secretion of TNF-α by isolated PBMCs showed significant decrease. This anti-TNF activity may be related to specific blockade of TNF-α secretion or to NF-κB pathway. It has been suggested that TNF-α regulates MCP-1 secretion via the activation of NF-κB (*76*). Yet, this was unlikely to occur in the case of Chios Mastic administration, given that MCP-1 concentration remained unaffected (Table 3). It is rather that the NF-κB pathway secondary to the decrease in TNF-α was not activated. A possible approach would be via the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. By blocking HMG-CoA reductase on human monocytes, cells reduce the production of TNF-α (*77*).

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Table 3. Effect of Chios Mastic oral administration in sera and PBMCs from patients with Crohn's disease

Sera	*Before*	*After*
IL-6 (ng/mL)	0.02±0.01	0.007±0.003*
TNF-α (ng/mL)	0.03±0.01	0.01±0.005
MCP-1 (ng/mL)	0.1±0.04	0.07±0.02
CRP (mg/mL)	40.3±13.1	19.7±5.5*
TAP (mmol/L)	0.15±0.09	0.57±0.15*
PBMCs	*Before*	*After*
IL-6 (ng/mL)	0.62±0.13	0.52±0.18
TNF-α (ng/mL)	2.1±0.9	0.5±0.4*
MCP-1 (ng/mL)	3.0±1.1	1.7±1.0
MIF (ng/mL)	1.2±0.4	2.5±0.7*
GSH (μmol/L)	34.0±15.8	56.6±10.3

* Asterisk points out statistically significant differences before and after treatment ($P < 0.05$).

Macrophage migration inhibitory factor (MIF) was originally described as an inhibitor of migration and chemotaxis of monocytes/macrophages. Suppressed secretion of MIF in mononuclear cells derived by patients with Crohn's disease compared to the respective levels in healthy subjects indicates that monocytes are sensitized to chemotaxis. Increased secretion after treatment with Chios Mastic points to the inhibition of monocyte chemotaxis (Table 3). The significance of this is that migration of chemokine or peptide or nonpeptide stimulated monocytes and differentiation to macrophages into the site of inflammation is limited and inflammation is regulated.

A steadily increasing number of research protocols has recently been developed and have contributed greatly to important advances in our current understanding of the immunological regulation by Chios Mastic.

Regarding toxicity and acceptable dose, orally administered mastic of 521 mg/kg/day apparently enhanced preneoplastic lesion in a rat liver medium-term carcinogenesis bioassay, while 52.1 mg/kg/day was a non-promoting dose. The latter dose is almost equivalent to 2.9 g/day/person with the average human body weight regarded as 60kg. Most mastic doses reported in human studies are around this level, e.g. 5 g/day/person (*68*), 4 g/day/person (*53*), 2.2 g/day/person (*73*, *74*), or 1 g/day/person (*50*). Favorable effects of mastic such as the anticarcinogenic potential could be achieved at relatively low doses without any toxicity (*78*, *79*).

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Greek Currants (Corinthian Raisins, *Vitis vinifera L.*)

Currants (Corinthian raisins, *Vitis vinifera L.,* Vitaceae Family) are dried vine fruits cultivated and processed since antiquity. Historical records confirm that grapes were dried under the sunlight since 1490 B.C. The climate in Greece was ideal for the growth of grapes, thus Greece became one of the main commercial markets for currants. The Greek vineyards produce different varieties of grapes, which are very competitive products in global market. Nowadays currnats represent about 8-10% of dried vine products production; other dried vine fruits varieties are the raisins – produced mainly in California and in some other mild climate countries – and the Sultanas – produced mostly in Turkey, Iran, Afganistan, South Africa, Australia, Chile, but also in Greece. Corinthian currants – produced from a special type of black grapes – are small sun-dried berries, coloured black to dark blue, and produced almost exclusively in Southern Greece. Based on the applied agricultural practices, the product properties, and the degree of product uniformity and cleanness, currants are classified in two main quality categories A and B. In the highest quality category A there are two subcategories, i.e. Vostizza and Gulf currants, the former considered as the superior quality one and holding a PDO name. Both Vostizza and Gulf currants are produced exclusively in North Peloponesse in the south of Greece while the Provincial currants (quality category B) are produced in Western Peloponesse and in two Ionian islands Zakynthos (Zante) and Cephalonia.

Nutritionally, raisins are perfect alternative sweeteners, high in fiber, complex carbohydrates, and minerals and vitamins necessary for vitality. A portion of 40g contains usually 28-32g of sugar - mainly fructose -, 2g of fibers, very small quantities of protein (usually 1g), sodium (roughly 10mg), calcium and iron; its energy content is 110-140kcal. Greek currants content in potassium is very high – usually 310mg that corresponds to approximately 10% of daily need in humans. With respect to the dried vine products polyphenol content and antioxidant activity very limited literature data exist. In the study of Karadeniz et al. (*80*) the polyphenol composition of sun-dried, dipped, and golden raisins obtained from Thompson seedless grapes (*Vitis Vinifera L.* cv. sultanina) was reported. Chiou et al. (*81*) reported the content in total and simple individual polyphenols of the several currants subcategories, while their antioxidant capacity in terms of scavenging the DPPH$^{\bullet}$ free radical was also assessed. Currants and Greek originated sultanas total polyphenol content was also reported by Kaliora et al. (*82*). Additionally in this latter study the *in vitro* antioxidant, antiatherogenic, and anticancer activity of the products was studied.

Antioxidant Activities of Currants

The antioxidant capacity of currant methanol extracts as screened by the DPPH$^{\bullet}$ radical scavenging assay was found similar for all three cultivars – Vostizza, Gulf, and Provincial – in the study of Chiou et al (*81*) while in the study of Kaliora et al (*82*) the decreasing order of scavenging activity was Gulf>Cretan sultanas>Provincial>Vostizza.

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Polar extracts from currants and sultanas inhibited the chemically-induced LDL oxidation and increased the levels of antioxidant GSH (*82*). oxLDL is associated with the pathogenesis of atherosclerosis, a key early stage of CVD, hence, the significance of this activity is evident. The effect on LDL was correlated to the polyphenol content, meaning that this effect could be attributed to the polyphenols as well. Polyphenols have been found to act against LDL oxidation, and their antioxidant capacity is related to their chemical structures. oxLDL is primarily responsible for GSH depletion creating an oxidizing environment required for γ-GSC induction and compensatory GSH synthesis (*63*, *83*). A product from LDL oxidation namely tBHP, is responsible for GSH decrease in PBMCs. Glutathione (GSH) is modulated in disorders caused by free radical attack (*84*). Glutathione the most abundant antioxidant in cells is found predominantly in two redox forms: reduced and oxidized. Its protective action is based on the oxidation of the thiol group of its cysteine residue with the formation of GSSG, which in turn is catalytically reduced back to the thiol form by gluatathione reductase. Measurement of the GSSG level or determination of the GSH/GSSG ratio, is a useful indicator of oxidative stress and can be used to monitor the effectiveness of antioxidant intervention strategies. No difference was observed in intracellular GSSG with currant extract while elevated levels of total GSH was observed when pre-treating PBMC with the extracts (*82*). This shows that incubation of cells with the plant extracts possibly induces γ-glutamylcysteine synthetase. Ghibelli et al (*85*) have shown that GSH decrease occurs during cell death through a physiological process, i.e via physiological carriers responsible for GSH efflux. When inhibiting the carriers, they not only observed GSH restoration, but also reduced cell death, thus proving that decrease in GSH is an intrinsic part of cell death signalling, a necessary step to trigger the events of apoptosis. Exposure of cells to tBHP resulted in decrease of GSH and decrease in cell survival. Also, cell survival was found to be correlated to total polyphenol content and to antioxidant activity as to LDL oxidation, showing that inhibition of oxidative stress triggered cytotoxicity is owed to the antioxidant activity of the contained polyphenols.

Because part of an agent's potential to protect against atherosclerotic plaque formation may be due to the protection of cells from apoptosis, the protective effect of currant extracts on oxidative stress- induced apoptosis was observed under a fluorescence microscope. The cells exposed to oxidizing agent exhibited features of programmed cell death; however, cells that were pre-treated with the extract had reduced characteristics of apoptotic cells. Their morphological observation was similar to this of the control cells. These data, obtained from a non quantitative assay, suggest that the extracts inhibited oxidizing agent-induced apoptosis.

Currants Anticancer Activity

Gastric cancers, 90% of which are adenocarcinomas, constitute a major cause of mortality both in developed and developing countries, because currently available chemotherapeutic regimens are not very effective resulting in high recurrence rates and poor survival. Gastric cancer is recognized as a multifactorial disease. The pathogenesis of carcinogenesis embodies genetic factors, infection with *Helicobacter pylori*, environmental factors and lifestyle factors, including dietary habits. Preventive dietary strategies offer the best opportunities for control of the disease. To this end, research on natural products defensive against stomach cancer cell proliferation is of intense interest. High molecular weight fractions of tea have been found to induce apoptosis in stomach cancer MKN-45 cells (*86*). Resveratrol suppressed both synthesis of DNA and generation of endogenous O_2^- but stimulated nitric oxide synthase (NOS) activity (*87*). Tea theaflavins have been shown to inhibit the growth of gastric cancer MKN-28 cells (*88*). Phenolic compounds isolated from sweetpotato leaves suppressed the proliferation of a stomach cancer cells Kato III (*89*).

The methanol extracts obtained from currants of different origin in Greece and sultanas exhibited gastric cancer preventive efficacy by limiting cell proliferation and suppressing ICAM-1 levels, both at the protein and at the mRNA levels, in gastric adenocarcinoma cells (AGS cell line). Differences in the activity of different qualities of currants were probably due to both the geographic origins and the different qualities. Inter–group differences were statistically significant for total polyphenol content, meaning that total polyphenols differ in products of different geographical origin.

The mechanisms of chemoprevention may be several including effects on cellular differentiation/cell cycle, apoptosis, and activation or deactivation of various enzyme systems such as the phase I or II biotransformation enzymes or antioxidant action. Protocatechuic acid and caffeic acid inhibited the growth of AGS cells through the induction of apoptosis associated with two signalling pathways; one was the activation of p38 signalling and the other was the stabilization of p53 (*90*). Currant and sultana extracts inhibited the proliferation of human gastric carcinoma cells through triggering apoptosis, rather than necrosis (*82*). A common feature of malignant cells is their ability to proliferate without restraint while apoptosis is a primary mechanism for the chemoprevention of cancer. Therefore, it is of interest to identify active compounds or mixtures of compounds from foods with apoptosis-inducing activity against cell lines.

A causal relation between inflammation and cancer has been proposed by many research groups, suggesting that cancers arise at regions of chronic inflammation (*91*). In chronic inflammation, proinflammatory cytokines, such as TNF-a, are induced and activate transcription factors, such as NF-κB and activator protein-1 (AP-1). Transcription factors, in turn, modulate the levels of inflammatory cytokines, such as IL-8, as well as intercellular adhesion molecules, such as ICAM-1, which is highly expressed in the stomach.

ICAM-1 protein and mRNA levels were decreased in the presence of currant extracts. ICAM-1 expression is dramatically increased at sites of inflammation, providing important means of regulating cell-cell interactions and thereby presumably inflammatory responses. Cancer cells with high ICAM-1 expression show a high invasive and metastatic potential along with the progression of the tumor.

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In patients with gastritis gastric mucosal levels of IL-8 correlate with histological severity (*92*). In gastric cancer, IL-8 induces expression of adhesion molecules such as ICAM-1 (*93*). Expression of IL-8 gene is primarily controlled at the transcriptional level. NF-κB and AP-1 are inducible transcription factors and their binding sites are found in the promoter region of the IL-8 gene (*94*). In gastric cancer cell lines, synchronized action of NF-κB and AP-1 seems important in the expression of IL-8 in response to cytokine stimulation (*95*). Contrary, to decrease in ICAM-1 protein and mRNA levels, IL-8 protein or mRNA levels remained unchanged in the presence of currant extracts. ICAM-1 expression is regulated by NF-κB transcription factor solely (*96*). It is rather that the extracts regulate gastric inflammation via the decreased activation of NF-κB, but not of AP-1.

Polyphenols in Currants

Chiou et al. (*81*) reported the total polyphenol content of the three currants sub-varieties – Vostizza, Gulf, and Provincial – in the range 155 - 246 mg GAE / 100g (mean 191±26 mg GAE/100g) with Provincial currants containing a rather higher total phenolic content than Vostizza and Gulf ones. Kaliora et al (*82*) have found similar total phenolic values for Vostizza and Provincial (from Messinia) currants; both these currants sub-varieties have been shown to contain considerably higher total phenolics than Sultanas obtained from the south of Greece (Cretan island). In this latter study Gulf currants have been reported to contain similar total phenolic content with that of Sultanas and considerably lower than the one reported by Chiou et al. (*81*).

Grapes are a rich source of polyphenols (*97*) ranging from simple compounds to complex tannin-type substances. Phenolic acids, stilbene derivatives, and flavonoids are among the several classes of polyphenolic compounds present in grapes; grape polyphenols can also be found in the form of tartaric acid esters or glycosylated. With respect to the dried vine products Chiou et al. (*81*) studied currants content in simple polyphenols; data reported are summarized in Figure 4.

The polyphenolic profile of the several currant sub-varieties was not differentiated, with the exception of the absence of 3,4-di-hydroxy-phenylacetic acid from the Vostizza samples. Seventeen polyphenol species were identified in Gulf and Provincial currants and 16 in Vostizza ones. Total simple polyphenols ranged from 4.81±0.99 mg /100 g in Vostizza to 6.71±2.03 mg /100 g in Provincial currants (mean 5.60±1.00 mg /100 g). In all studied samples benzoic acids predominated, while among them vanillic acid was the main polyphenol species in all cases. Resveratrol – a stilbene with anticarcinogenic properties – was present at mean concentration 0.19±0.07 mg / 100 g. Though not quantified, the presence of catechin and epicatechin and the triterpenic oleanolic acid was also reported.

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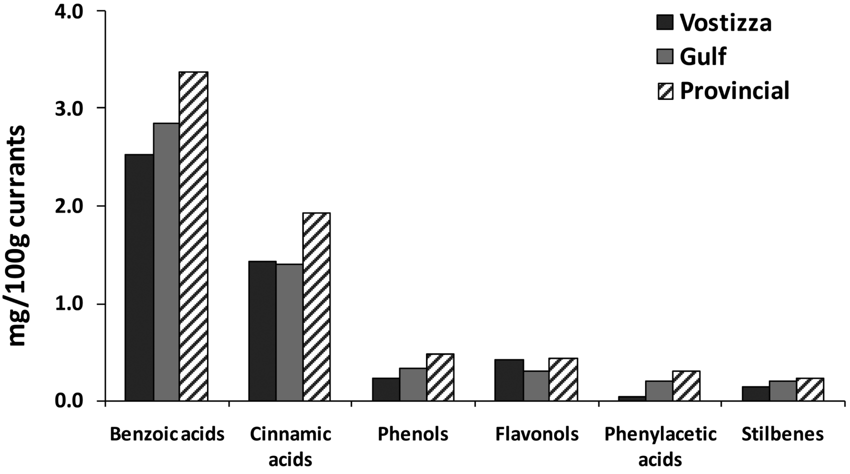

Figure 4. Benzoic acids (vanillic acid, gallic acid, syringic acid, p-hydroxybenzoic acid, protocatechuic acid), cinnamic acids (cinnamic acid, phloretic acid, p-coumaric acid, ferulic acid, caffeic acid), phenylacetic acids (p-hydroxy-phenylacetic acid, 3,4-di-hydroxyphenylacetic acid), phenols (tyrosol, vanillin), flavonols (quercetin, kaempferol) and stilbenes (resveratrol) present in the three currants sub-varieties.

Conclusions

The principal aspects of the most healthful nutritional recommendation namely Mediteranean Diet include among others high consumption of legumes, fruits, as well as medicinal plants such as herbs or Chios Mastic. Legumes are particularly characteristic of the Mediterranean diet and their nutritional value is considered very high, while additionally identification of different phenolic and terpenic compounds in them highlights their potent role as health promoting food components.

Herbs infusions are rich in polyphenolic compounds which together with their essential oils' components are expected to exert beneficial action towards several denegerative diseases.

Mastic is widely used as a food additive and exerts beneficial health effects attributed to several bioactive constituents. The cardioprotective and immunoregulating effects established *in vitro* and *in vivo* need to be further studied in larger cohorts, and may aid to design new therapies for prevention in atherosclerosis and other related cardiovascular diseases, as well as in prevention in inflammatory bowel diseases.

The *in vitro* antioxidant/antiatherogenic effectiveness of currants, owed mainly to the contained polyphenols, and their chemopreventive effect are of immense interest in the development of strategies to prevent or delay cardiovascular events and several forms of cancer.

In future, more than just nutritional items, such natural products may be considered as factors with priorities in medical applications.

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List of Abbreviations

AAE	Ascorbic acid equivalents
AP-1	Activator protein-1
CD36	Cluster of Differentiation 36
CRP	C-Reactive protein
DNA	Deoxyribonucleic acid
DPPH$^{\bullet}$	2,2-Diphenyl-1-picrylhydrazyl free radical
FRAP	Ferric ion reducing antioxidant power
GAE	Gallic acid equivalents
G-CSF	Granulocyte-colony stimulating factor
γ-GSC	γ-Glutamyl cysteine synthetase
GSH	Glutathione
GSSG	Glutathione disulfide
ICAM-1	Inter- Cellular Adhesion Molecule-1
IFN-γ	Interferon-gamma
IL-	Interleukin-
LDL	Low density lipoprotein
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
MIF	Macrophage migration inhibitory factor
mRNA	Messenger Ribonucleic acid
NF-κB	Nuclear factor-kappa Beta
NO	Nitric oxide
PBMC	Peripheral Blood Mononuclear Cells
PDGF	Platelet-derived growth factor
SGOT	Serum glutamic oxaloacetic transaminase
SGTP	Serum glutamic pyruvic transaminase
TAP	Total antioxidant potential
TBARS	Thiobarbituric acid
tBHP	tert-Butyl hydroperoxide
TE	Trolox equivalents
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
VCAM-1	Vascular Cell Adhesion Molecule-1

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Chapter 14

Hypoglycemic Bioactives from a Traditional Herb

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Malaysia is a country rich with its biodiversity heritage. Through generations of practice, the multi-ethnic communities have relied upon the tropical rainforest for medical relief. Nonetheless, few scientific reports supported it's usage. The aim of this study is to evaluate the efficacy of *Brucea javanica* Merr (Simaroubaceae) for diabetes treatment. The seeds were extracted with methanol and partitioned with increasing polarity solvents. Through bioactivity guided fractionation, bruceines D and E exhibited hypoglycaemic activity. Normoglycemic mice administered intraperitoneally (*i.p.*) with 0.5 mg/Kg of bruceines D and E exhibited blood glucose lowering of 31.4 ± 11.3 % and 40.5 ± 12.9 %, respectively. Both bruceines D and E also exhibited blood glucose lowering of 88.0 ± 2.9 and 50.4 ± 19.9 % in streptozotocin induced diabetic rats administered with 1 mg/Kg (*i.p.*). This was comparable to diabetic rats fed (*i.p.*) with positive control, glibenclamide, with reduction of 71.5 ± 18.7 %. In conclusion, this study supported the use of *B.javanica* seeds by diabetes patients.

Keywords: *Brucea javanica*; quassinoids; hypoglycemia

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Introduction

Malaysia is located in the equatorial region and the climate is humid with sunshine throughout the year promoting the diversity of flora and fauna in the rainforest. Through generations of practice, the multiethnic community in Malaysia have relied on the vast diversity of the rainforest for medical relief. Diabetes mellitus is a chronic metabolic disorder in association with the endocrine system and one of the leading public health concerns in both developed and developing countries. The prevalence of diabetes is estimated to double globally from 143 million in 1997 to more than 300 million in 2030 (*1*, *2*). There are two main types of diabetes mellitus, insulin-dependent diabetes mellitus (IDDM) or type 1 and non-insulin dependent diabetes mellitus (NIDDM) or type 2. Type 1 diabetes mellitus is characterized by disability to produce insulin due to pancreatic islet beta-cell destruction and mostly occurs in children and young adults and the more common type 2 diabetes mellitus is characterized by insensitivity of cells to insulin.

Clinically, the drugs used in the treatment of type 2 diabetes are mainly divided into insulin secretagogues such as sulfonylureas (glibenclamide and tolbutamide) and meglinitides (repaglinide and nateglinide), insulin sensitizers such as thiazolidinediones and metformin. These drugs are employed to reduce the level of blood glucose but produces side effects such as lactic acid intoxication and gastrointestinal upset (*3*).

Natural products may provide suitable drug candidate for the treatment of diabetes. Herbs with hypoglycemic effects have often been used in folk medicine or by traditional healing practitioners for the treatment of diabetes (*4*). Crude extracts from the plants of the Simaroubaceae family, namely *Castela texana* (T. & G.) Rose, *Castela tortousa* Liebm. (*5*), *Picrasma crenata* (Vell.) Engler (*6*), *Ailanthus excels* (*7*) and *Eurycoma longifolia* Jack (*8*) have been reported to exhibit hypoglycemic effect.

The Simaroubaceae family consists of about 21 genera and 150 species of tropical trees. The trees from the genera of *Brucea* (*9*, *10*), *Ailanthus* (*11–13*), *Eurycoma* (*14–17*), *Simaba* (*18–20*), *Castela* (*21–23*) *and Picrasma* (*24–26*) are the most intensely investigated over the years. These trees are known for its bitter barks, woods and seeds mainly attributed to the content of quassinoids which displays a range of cytotoxic, antiviral, antiinflammatory and antiamoebicidal properties (*27*). The quassinoids, quassinoid glycosides, alkaloids and lignans are groups of chemical constituents from the Simaroubaceae family.

Brucea is one of the genera under the Simaroubaceae family. It has been widely investigated since 1960's and some of the species under this genus are *Brucea javanica* (L.) Merr., *Brucea amarissima* (Lour.) Desv. ex Gomes and *Brucea sumatrana* Roxb. *B.javanica* grows in tropical areas distributing from Southeast Asia (Thailand, Vietnam, Indonesia, Malaysia, Papua New Guinea and Philippines) to Northern Australia.

Some of the quassinoids from *B. javanica* are bruceines A-F **(1-6)**, bruceines H and J **(7-8)** (*28–32*), bruceantin **(9)** (*33*), bruceantarin **(10)**, bruceantinol **(11)** (*34*), bruceolide **(12)** (*35*), brusatol **(13)** (*36*), desmethyl-brusatol **(14)** (*37*), (Figure 1) dehydrobruceantinol **(15)** and dehydrobrusatol **(16)** (*38*), dehydrobruceines A

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(17) (*39*) and B **(18)** (*40*). Quassinoid glycosides from this species are bruceosides A-C **(19-21)** (*41–43*), bruceosides D-F **(23-25)** (*44*), javanicosides A **(22)** (*45*) and B-F **(26-30)** (*46*) (Figure 2).

According to Liu *et al.* (*47*), indole alkaloids of the β-carboline and canthin-6-one have been identified from a number of Simaroubaceae species but, only two alkaloids have been reported occurring in *Brucea* species, namely, 10-methoxycanthin-6-one **(31)** and 10-hydroxycanthin-6-one **(32)** (*48*, *49*) (Figure 3). Most of the chemical constituents studied, exhibited cytotoxic effects. The objective of this study is to identify and evaluate bioactives responsible for its hypoglycemic effect.

Materials and Methods

The Plant

The plant was authenticated by Emeritus Professor Dato' Dr Abdul Latiff Mohamed from Universiti Kebangsaan Malaysia, Bangi, Malaysia. A voucher specimen (FP/UiTM/BJ/01/05) was deposited at the Faculty of Pharmacy, Universiti Teknologi MARA, Malaysia.

Extraction

The ground seeds were soaked overnight in 80 % (v/v) methanol in deionized water at 40 ± 1 °C. Aqueous methanolic extract was filtered and dried *in vacuo* at 40 ± 1 °C. Dried methanolic extract was suspended in deionized water and sequentially partitioned with *n*-hexane, chloroform and 1-butanol to afford the hexane, chloroform, butanol and residual soluble fractions. Each fraction was dried with a rotary evaporator (Buchi, Switzerland) and dried extracts were stored at 4 °C until further used.

Isolation of Pure Chemical Constituents

The bioactive butanol extract afforded four pooled fractions, namely BJA-D, after subjected to normal phase (Merck, 0.063-0.200 mm) open column chromatography with increasing ratio of methanol in chloroform (9:1, 7:3, 5:5 and 0:10). Further fractionation of bioactive BJB afforded two pure chemical constituents, namely bruceine D **(4)** and E **(5)**. The structures of these two chemical constituents were structurally elucidated using nuclear magnetic resonance (NMR), infrared (IR), mass (MS) and ultraviolet-visible spectrometry and compared with reported data (*50*).

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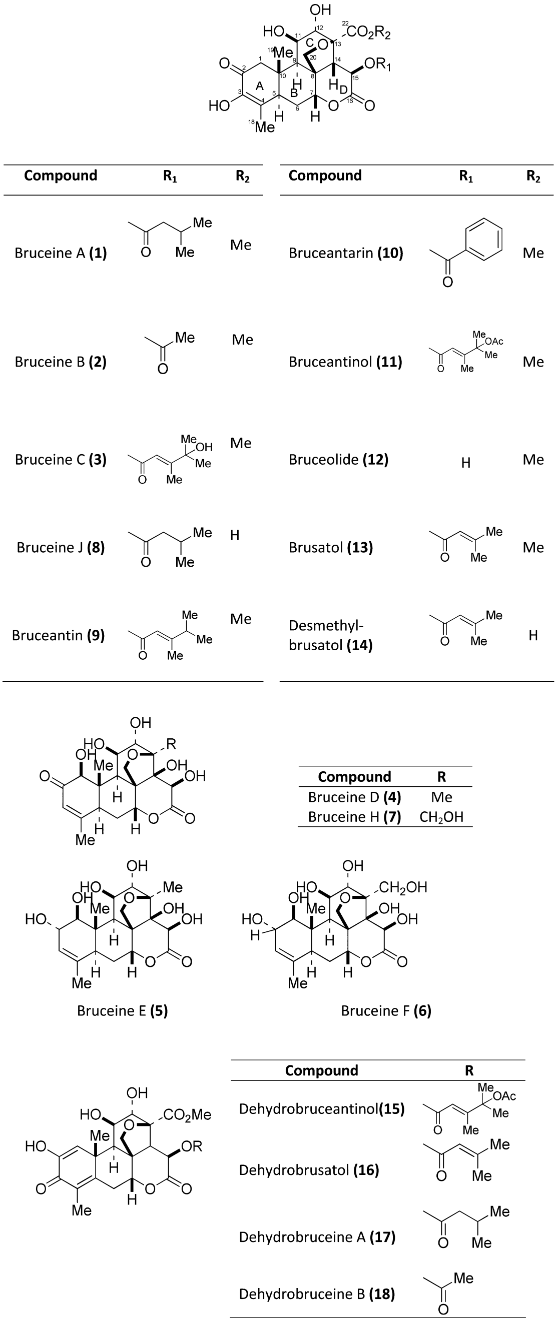
Compound
R1
R2
Bruceine A (1)
Me
Bruceine B (2)
Me
Bruceine C (3)
Me
Bruceine J (8)
H
Bruceantin (9)
Me
Bruceantarin (10)
Me
Bruceantinol (11)
Me
Bruceolide (12)
H
Me
Brusatol (13)
Me
Desmethyl-brusatol (14)
H
Compound
R
Bruceine D (4)
Me
Bruceine H (7)
CH2OH
Bruceine E (5)
Bruceine F (6)
Compound
R
Dehydrobruceantinol(15)
Dehydrobrusatol (16)
Dehydrobruceine A (17)
Dehydrobruceine B (18)

Figure 1. Quassinoids from B. javanica.

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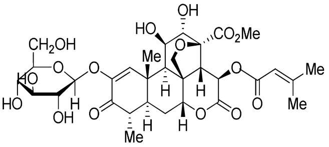
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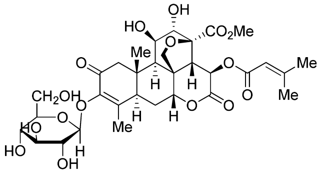
HO
CO2Me
CH2OH
Me
O
Me
HO
HO
H
H
H
OH
O
O
O
O
Me
Me
H

Bruceoside A **(19)**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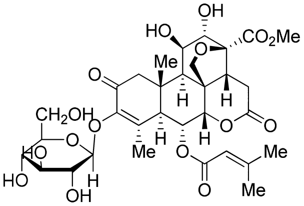
CO2Me
Me
O
Me
CH2OH
Me
HO
HO
H
OH
Me
H
H

Bruceoside B **(20)**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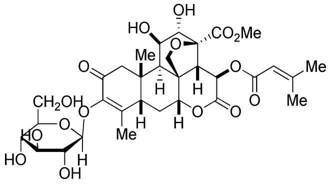
CO2Me
Me
O
CH2OH
HO
HO
H
OH
Me
H
H
Me
O
Me

Bruceoside C **(21)**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CO2Me
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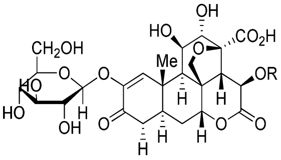
Me
CH2OH
Me
HO
HO
H
OH
H
H

Javanicoside A **(22)**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CH2OH
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OR
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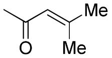
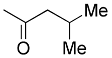
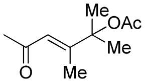
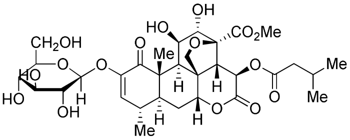
HO
H
OH
O
O
H
H
H

Compound	R
Bruceoside D **(23)**	
Bruceoside E **(24)**	
Bruceoside F **(25)**	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CH2OH
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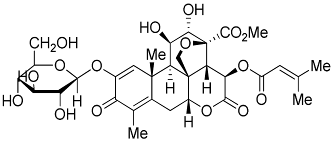
Me
Me
HO
HO
H
OH
Me
H
H
Me

Javanicoside B **(26)**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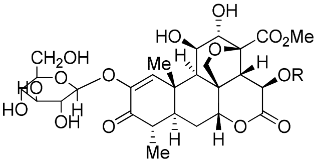
HO
CO2Me
CH2OH
Me
Me
HO
HO
H
OH
Me
H
H
Me

Javanicoside C **(27)**


OH
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CO2Me
CH2OH
Me
H
H
OR
HO
HO
H
OH
Me
H
H

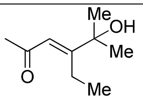
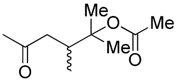
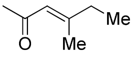
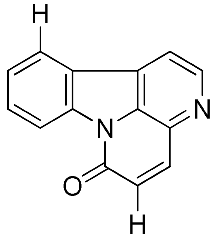
Compound	R
Javanicoside D **(28)**	
Javanicoside E **(29)**	
Javanicoside F **(30)**	

Figure 2. Quassinoid glycosides from B. javanica.



Compound	R
10-methoxycanthin-6-one **(31)**	OMe
10-hydroxycanthin-6-one **(32)**	OH

Figure 3. Alkaloids from B. javanica.

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Normoglycemic Mice

Normoglycemic male *Mus musculus* mice, weighed between 25 and 30 g were housed in an ambient temperature of 25 ± 1 °C with a relative humidity of 65 ± 5 % and 12 hour light/dark cycle and given water *ad libitium* and pelletized food (GoldCoin Enterprise, Malaysia). Mice were acclimatized for one week and fasted for 12 hours. All experiments were conducted after approval from the Local Animal Ethical Committee. Six groups of mice in groups of 10 each were prepared. Group 1 was the control group and fed with the solvent only. Group 2 was fed with 3 mg/kg of the positive control, glibenclamide. Groups 3 and 4 were fed with either 0.5 or 2.0 mg/Kg of bruceine D **(4)**. Groups 5 and 6 were fed with either 0.5 or 2.0 mg/Kg of bruceine E **(5)**. Blood was withdrawn from the tail vein immediatedly after the administration of bioactives. Blood glucose was measured at 0, 4 and 8 hours with a glucometer (Ascensia Elite, Bayer Corporation, USA).

STZ-Induced Diabetic Rats

Freshly prepared streptozotocin (STZ) solution in 0.1 M citrate buffer with pH 4.5 was administered by a single intraperitoneal injection to the fasted rats at a dose of 60 mg/kg BW. The rats were then provided with 5 % glucose solution to prevent death owing to oxidative stress. The rats with blood glucose concentration above 13.3 mmol/l or 250 mg/dl were subjected to further experiment (*51*). Practically, 20 STZ induced diabetic rats were divided into four groups of 5 each with group 1 receiving 0.2 %w/v PEG 3000 solution as the vehicle. The other three groups received 1 mg/kg BW of STZ induced diabetic rat with either bruceine D **(4)** or bruceine E **(5)** through intraperitoneal administration. The blood glucose lowering effects of **(4)** and **(5)** in STZ induced diabetic rats were determined from the blood withdrawn from the tail vein at 0, 4 and 8 hour. Glibenclamide, the positive control was administered intraperitoneally at a dose of 3 mg/kg BW of STZ induced diabetic rat.

Statistical Analysis

All data are expressed in mean and standard deviation. Two-way ANOVA by SPSS statistical package version 14 is used to evaluate all data with the level of significance set at $p < 0.05$.

Results and Discussions

Quassinoid and quassinoid glycosides from *B.javanica* exhibited significant cytotoxic effect against various cancer cell lines. The quassinoid, bruceantin **(9)** is the most intensely investigated quassinoid subjected to Phase I and II clinical trials but was terminated due to lack of efficacy (*52*). Quassinoids, namely, bruceines A-C **(1-3)**, bruceantinol **(11)**, bruceolide **(12)** and brusatol **(13)** are some of the quassinoids exhibiting cytotoxic effect against the human epidermoid carcinoma of the nasopharynx (KB) cells (*29*, *53*). Bruceine D **(4)** also exhibited marked *in vitro* activity in human promyelocytic leukemia (HL-60) cells (*54*). Bruceantin **(9)**, bruceantarin **(10)** and bruceantinol **(11)** were significantly cytotoxic against drug-resistant cell lines, KB-VIN or KB-CPT. Presence of the ketone moiety in ring A and the position of the conjugated enone system are essential for this activity (*55*).

Quassinoid glycosides have also been studied for its biological activity as antileukemic agents. Bruceosides A **(19)** and B **(20)** were the first two isolated quassinoid glycosides to possess antileukemic activity (*42*). Bruceoside C **(21)** exhibited a strong cytotoxic effect against KB cells, central nervous system carcinoma (TE-671 or human medulloblastoma) as well as murine lymphocytic leukemia (P-388) cells with the ED_{50} values less than 0.1, 0.29 and 5.11 µg/ml, respectively (*43*). National Cancer Institute, USA, has evaluated three quassinoid glycosides, bruceosides D **(23),** E **(24)** and F **(25)**, for their cytotoxicity effects against various cancer types. These quassinoid glycosides were selectively cytotoxic to certain tumor cell lines of leukemia, melanoma, non-small cell lung, colon, central nervous system and ovarian cancers with the log GI_{50} values ranged from -4.14 to -5.72 (*44*). Javanicoside B **(26)** exhibited a moderate cytotoxicity effect against the P-388 cells with the IC_{50} value of 5.6 µg/ml, while javanicosides C-F **(27-30)** exhibited no activity against the P-388 tumor cells with the IC_{50} values of greater than 18, 89, 16 and 50 µg/ml, respectively (*46*).

Other activities exhibited by both quassinoid and quassinoid glycosides were inhibition against Epstein-Barr virus antigen (*56*), *Plasmodium falciparum* (*39*), *Entamoeba histolytica* (*57*) and HIV (*58*). Though Simaroubaceae plants have been reported (*5–8*) to exhibit hypoglycemic effect, chemical constituents responsible for it's effect have not been reported.

Traditionally, the seeds of *B.javanica* is consumed by diabetic patients, thus the seeds were extracted in aqueous methanol and subjected to hypoglycemic evaluation. Through bioactivity-guided-fractionation, two bruceines, namely, bruceine D **(4)** and bruceine E **(5)** were isolated. Both the doses of bruceine D **(4)**, 0.5 mg/kg and 2 mg/kg, exhibited significant blood glucose reduction after 8h of administration. Only higher concentration of bruceine E **(5)** of 2 mg/kg exhibited significant blood glucose reduction after 8h of administration. Administration

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of 0.5 mg/kg body weight (BW) of bruceine D **(4)** significantly reduced blood glucose to 40.5 ± 12.9 % following 8h of administation (Figure 4). Increasing the dosage to 2 mg/kg BW of bruceine D **(4)**, increased the blood glucose reduction to 47.6 ± 2.8 %. Similarly, mice administered with 2 mg/kg of bruceine E **(5)** exhibited significantly higher blood glucose reduction of 42.3 ± 16.6 % compared to 31.4 ± 11.3 % blood glucose reduction in mice administered with 0.5 mg/kg dosage (Figure 5). Thus, a higher dose of bruceine E **(5)** is required to exhibit equivalent blood glucose reduction effect as bruceine D **(4)**.

Both bruceines D **(4)** and E **(5)** exhibited higher blood glucose reduction in STZ induced diabetic rats. Rats administered with 1 mg/kg BW of either bruceine D **(4)** or E **(5)** exhibited a blood glucose reduction of 88.0 ± 2.9 % and 50.4 ± 2.0 %, respectively (Figure 6). Both the blood glucose reduction was comparable to the rats administered with the positive drug, glibenclamide. The chemical structure of bruceine D **(4)** differs from bruceine E **(5)** at the C_2 position with the presence of a ketone instead of a hydroxyl moiety. Similar to the cytotoxic effects of quassinoids (*55*), the presence of the ketone moiety at C_2 may be a structural requirement for the hypoglycemic effect. Both the bruceines D **(4)** and E **(5)** may act as an insulin secretagogue on surviving β-cells of islets of Langerhans to release more insulin.

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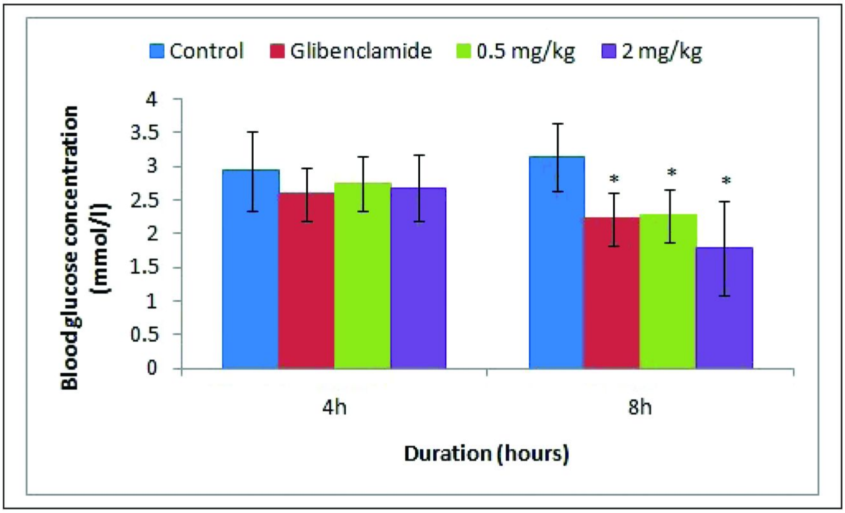

*p<0.05, in relation to control

*Figure 4. Blood glucose reduction in normoglycemic mice (n=10) administered with bruceine D **(4)**. (see color insert)*

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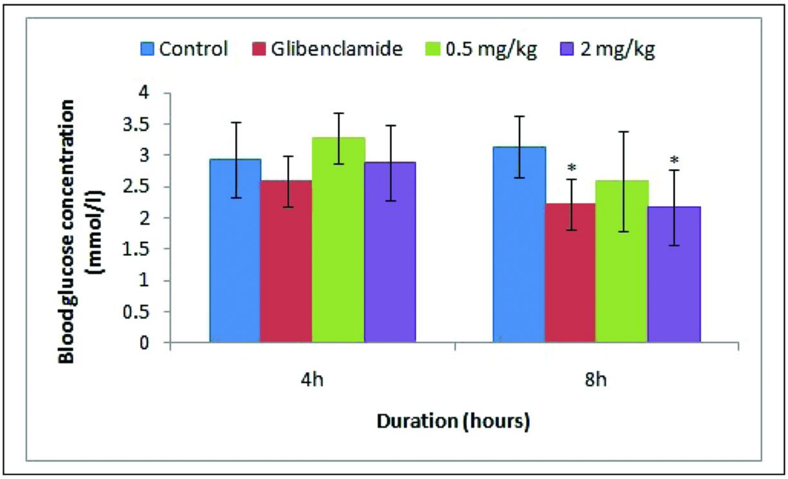
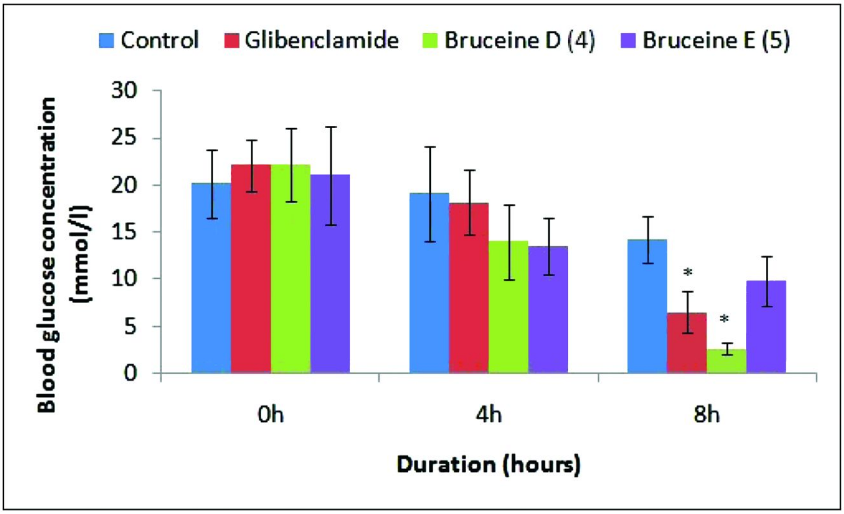


*$p<0.05$, in relation to control

*Figure 5. Blood glucose reduction in normoglycemic mice (n =10) administered with bruceine E **(5)**. (see color insert)*



*$p<0.05$, in relation to control, Data are from reference 59.

*Figure 6. Blood glucose reduction in STZ induced diabetic rats (n = 5) administered with 1 mg/kg of bruceine D **(4)** or bruceine E **(5)**. (see color insert)*

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Conclusion

The quassinoids, namely bruceine D **(4)** and E **(5)** exhibited blood glucose reduction in both the normoglycemic mice and streptozotocin induced diabetic rats. Thus, this is the first study supporting the usage of *B.javanica* seeds in traditional medicine for the treatment of diabetes.

Acknowledgments

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Chapter 15

Sugars, Acids, and Phenolic Compounds in Chinese Hawthorn (*Crataegus* spp.) Fruits of Different Origins

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Sugars, sugar alcohols, fruit acids and phenolic compounds were analysed in fruits of different species and cultivars of hawthorn (*Crataegus* spp.). Glucose and fructose were the major sugars commonly present in hawthorn, whereas sucrose was found only in some varieties. The major fruit acids were citric, quinic and malic acids. Hawthorn fruits were rich in sorbitol and *myo*-inositol. The major phenolic compounds in hawthorn were hyperoside, isoquercitrin, other quercetin glycosides, ideain, epicatechin, B-type procyanidins with polymerization index of 2-6 and their glycosides. Significant compositional variation existed among species, cultivars and varieties of hawthorn suggesting the need for selection of optimal raw materials for specific applications.

Introduction

Nutraceuticals and functional foods play an important role in the management of lifestyle-related health problems in modern societies. Compositional investigation on new potential raw materials is crucial for the development of nutraceuticals and functional foods. Fruits of hawthorn (*Crataegus* spp.) have been traditionally used both as food and as medicine in China (*1*, *2*). In western countries, these fruits are increasingly popular as new raw materials for food and food supplements with targeted physiological effects (*3*, *4*). Scientific evidence suggests beneficial effects of the fruits on sugar and lipid metabolism, cardiovascular health and immune functions (*5–10*). Current knowledge on the

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bioactive components and the mechanisms responsible for the health benefits is limited (*4*). The aim of the present work was to investigate the composition of hawthorn fruits with special focus on sugars, sugar alcohols, fruit acids and phenolic compounds important for the sensory properties and biological activities of the fruits. Fruits of different species, varieties and cultivars were compared.

Materials and Methods

Hawthorn Fruit Samples

Altogether twenty-two samples of hawthorn fruits were included in the study including ten cultivars of the variety *C. pinnatifida* var. *major*, eight cultivars of *C. brettschneideri,* three forms of *C. pinnatifida*, and one form of *C. scabrifolia.* Of these samples, the fruit sample of *C. scabrifolia* was collected from Kunming, Yun'nan Province, China in 2007, and the rest were collected from the Chinese National Fruit Germplasm Repository, Shenyang Hawthorn Garden (Shenyang, Liaoning Province, China) during 2007 and 2008. Table I presents a summary of the samples analyzed in the current study.

Table I. Hawthorn samples analyzed in the current study

C. pinnatifida var. major	*C. brettschneideri*	*C. pinnatifida*	*C. scabrifolia*
947	Caihong	Shanzha 1	Yun'nan shanzha
8321	Hongrou-shanlihong	Shanzha 2	
Dajinxing	Hongrou-shanzha	Shanzha 3	
Huixian-dahong	Jifu 1		
Jiangou 2	Jifu 3		
Mopan	Xinghong 2		
Qiujinxing	Zuofu 1		
Shandong-dajinxing	Zuofu 2		
Shen78201			
Zizhenzhu			

The fruits were picked as optimally ripe as determined by an experienced horticulturist based on the colour, structure and flavour of the fruits. The fruits were sliced and air-dried in a cool and shady place immediately after harvesting. The samples were stored in a desiccator in a cold room with avoidance of light. Before analyses, seeds were removed from dried hawthorn fruits. The seedless fruits were milled into powder in the presence of liquid nitrogen.

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Reference Compounds and Reagents

D-fructose, D-quinic acid, chlorogenic acid, and L-ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO). D-glucose, D-sorbitol, and the internal standard D-xylose (for sugars) were purchased from Fluka Chemie AG (Buchs, Switzerland). Malic acid and the internal standard L-tartaric acid (for acids) were purchased from Merck KGaA (Darmstadt, Germany). Sucrose, citric acid, and the internal standard D-mannitol (for sugar alcohols), methanol (HPLC grade) and formic acid were purchased from J. T. Baker B.V. (Deventer, Holland). *Myo*-inositol was purchased from Alexis Co. (Lausen, Switzerland). Hyperoside (quercetin-3-*O*-galactoside), isoquercitrin (quercetin-3-*O*-glucoside), indeain chloride (cyanidin-3-*O*-galactoside chloride), epicatechin and procyanidin B2 (epicatechin-(4β→8)-epicatechin, PA B2) were purchased from Extrasynthese (Genay, France).

Ethanol was from Primalco Oy (Rajamäki, Finland), and acetone (HPLC grade) and acetonitrile (HPLC grade) from VWR International Oy (Espoo, Finland). Tri-Sil HTP reagent was purchased from Pierce Chemical Co. (Rockford, IL). The reagent was composed of hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS) and pyridine (2:1:10).

Analysis of Sugars, Sugar Alcohols, and Fruit Acids

The sugars, sugar alcohols and fruits acids were extracted from the fruit powder with MilliQ water (powder/water, 1/100, w/v) and with the aid of ultrosonication (30 min) (*11*). After extraction, the sample was centrifuged, and a portion (3.0 mL) of the supernatant was taken, and xylose, mannitol, and tartaric acid were added (250 µL of water solution with concentration of 0.5 g/100 mL) as the internal standards for sugars, sugar alcohols and fruit acids, respectively. After filtration of the sample, a 50 µL portion was taken and silylized with Tri-Sil HTP reagent (*11*).

For identification of the compounds, the trimethylsily (TMS)-derivatives of the samples and the reference compounds were analyzed with a Shimadzu QP 5000 MSD GC-MS (Kyoto, Japan). The column used was DB-1MS (30 m L × 0.25 mm i.d. × 0.25 µm d_f) (J & W Scientific, Agilent, Folsom, CA) (*11*). The sugars, sugar alcohols and fruit acids were identified by comparing the retention times and the mass spectra of the sample peaks with those of the reference compounds. For quantitative analysis, the TMS-derivatives of sugars, sugar alcohols, and fruit acids were analysed with a Hewlett Packard 5890 Series II gas chromatograph (GC, Hewlett-Packard Co., Palo Alto, CA) equipped with a flame ionization detector (FID) and a Hewlett Packard 7673 auto-sampler (*11*).

Qualitative Analysis of Phenolic Compounds

Phenolic compounds were extracted from the seedless hawthorn fruit powder with 80% aqueous ethanol in an ultrasonicator bath (20 mL x 3, 15 min for each extraction). After the removal of ethanol, the extract was redissolved in 50% aqueous methanol and fractionated into 19 fractions on a polyamide column using sequentially MilliQ water, methanol and aqueous acetone as the eluting solvents (*12*). The fractions were analyzed with high performance liquid chromatography combined with diod array detection (HPLC-DAD) and high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS). The compounds were identified based on reference compounds, retention times, UV-absorption spectra and mass spectra.

The HPLC-DAD system (Shimadzu, Kyoto, Japan) consisted of a GT-154 vacuum degasser, two LC-10AT pumps, a SIL-10A automatic injector, a CTO-10A column oven, a SPD-M10A VP diode array detector (DAD), and a SCL-10A VP system controller. The system was operated using Class-VP 6.1 Workstation software. A Phenomenex Prodigy RP-18 ODS (*3*) column (5 μm, 250 × 4.60 mm, Torrance, CA) combined with a Phenomenex Prodigy guard column (5 μm, 30 × 4.60 mm, Torrance, CA) was used. The mobile phase consisted of water/formic acid/ (99.5/0.5, v/v) as solvent A and acetonitrile /methanol (80/20, v/v) as solvent B. The gradient programme is shown in Table II. The flow rate of the mobile phase was 1 mL/min, and the injection volume was 10 μL. Peaks were recorded at three different wavelengths: 280, 360 and 520 nm.

HPLC-ESI-MS analysis was carried in a positive ion mode using a Waters Acquity Ultra Performance LC system in combination with a Waters Quattro Premier mass spectrometer (Waters Corp., Milford, MA) equipped with an ion-spray interface. The capillary voltage was set to 4.0 kV, the cone voltage 22 V, and the extractor voltage 3 V. The source temperature was 150 °C and the desolvation temperature 300 °C. Mass spectra were obtained by scanning ions between *m/z* 200 and 900 and between *m/z* 900 and 2000 (*12*). The column for all HPLC analysis was at room temperature (20 °C)

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Table II. Mobile phase program during HPLC-DAD and HPLC-ESI-MS analysis

	0-5 min	*15 min*	*25 min*	*30 min*	*35 min*	*40 min*	*45 min*	*50 min*	*55 min*
A	90%	82%	82%	75%	75%	65%	40%	90%	90%
B	10%	18%	18%	25%	25%	35%	60%	10%	10%

Quantitative Analysis of Phenolic Compounds

The quantitative analysis of major phenolic compounds in aqueous ethanolic extracts of hawthorn fruits were performed with HPLC-ESI-MS in a positive ion mode using the selected-ion-recording (SIR) function (*13*). The ions of *m/z* 291 (nominal mass 291.3), 303 (303.2), 355 (355.3), 449 (449.4), 579 (579.5), 741 (741.7) and 867 (867.8) were monitored. The ions presented the base peaks in the mass spectra of epicatechin (291), hyperoside (303), isoquercitrin (303), chlorogenic acid (355), ideain (449), PA dimers (579), PA dimer-hexoside (741) and PA trimers (867). The instrumentation and the instrumental parameters were the same as described for the qualitative analysis. The quantification was carried out using an external standard method. The calibration curves of chlorogenic acid, ideain, hyperoside, isoquercitrin, epicatechin, PA B2 were prepared by analysis of commercial reference compounds. In addition, a PA dimmer (PA dimer II), a PA dimer-hexoside and a PA trimer (PA trimer II) were isolated from hawthorn fruit extract by preparative HPLC and used for preparation of calibration curves for quantification of these compounds in the hawthorn samples. The calibration curve of PA trimer II was used for quantification of all the PA trimers in the hawthorn samples.

Statistical Analysis

Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and Unscrambler 9.8 (Camo Process AS, Oslo, Norway). Differences in chemical composition among the species were analyzed using one-way analysis of variance (ANOVA) and with the Games–Howell and Student–Newman–Keuls (SNK) tests. Differences reaching a confidence level of 95% were considered significant. Pearson's correlation coefficient analysis was carried out to investigate the correlation between the contents of different phenolic compounds.

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Results and Discussion

Sugars, Sugar Alcohols, and Fruit Acids

Fructose and glucose were the major sugars commonly found in the fruits of all the species and cultivars analyzed (*11*). As shown in Figure 1, the content of fructose and glucose as well as the total content of the sugars and sugar alcohols were higher in the cultivars of *C. pinnatifida* var. *major* and *C. brettschneideri* than in the natural forms of *C. pinnatifida* and *C. scabrifolia*. Sucrose was found only in the fruits of *C. scabrifolia* and three cultivars *C. pinnatifida* var. *major,* 8321, Huixiandahong, and Shandongdajinxing, at levels of 24, 21, and 11 g/100 g dry mass (DM), respectively (*11*). Sorbitol was abundant in practically all the samples analyzed with the highest levels (11-16 g/100 g dry mass) found in the cultivars of *C. brettschneideri* and the lowest level in the fruits of *C. scabrifolia* (about 3 g/100 g dry mass) (Figure 1). *Myo*-inositol was found in the hawthorn fruit samples at levels of 0.1-0.2 g/100 g dry mass (Figure 1). The exceptionally high content of sorbitol and *myo*-inositol may have significance in the health effects of hawthorn (*14–16*).

Citric acid was the most abundant acid in all the samples except in *C. scabrifolia*, where the content of quinic acid exceeded those of citric and malic acids (*11*) (Figure 2). The sugar/acid ratio was higher in the cultivars of *C. pinnatifida* var. *major* and *C. brettschneideri* than in the natural forms of *C. pinnatifida* and *C. scabrifolia.* The highest sugar/acid ratios were found in the fruits of Jiangou 2 (13.2) and Shen78201 (11.6), two cultivars of *C. pinnatifida* var. *major* (*11*). Overall the hawthorn samples fell into sugar- and acid-rich groups. The cultivars Dajinxing, Jiangou2, Qiujinxing, Shen78201 and Zizhenzhu of *C. pinnatifida* var. *major* and all the cultivars of *C. brettschneideri* belong to the former group and the rest to the latter (*11*).

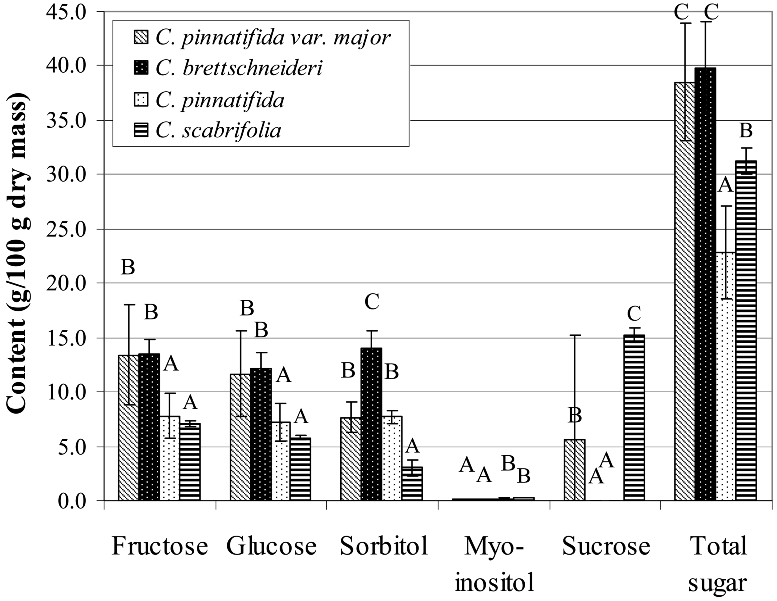

Figure 1. Content of sugars and sugar alcohols in hawthorn samples of different species and varieties. For each component, values not sharing common letters in the data labels differ significantly from each other (P < 0.05) (11).

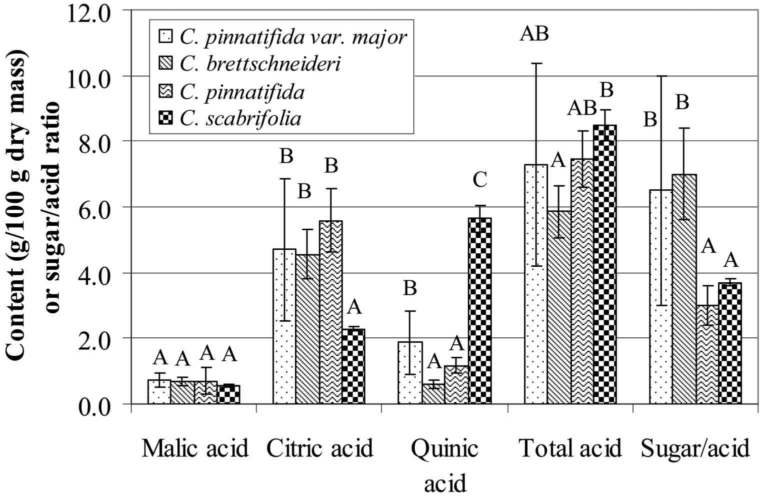

Figure 2. Content of fruit acids and sugar/acid ratio in hawthorn samples of different species and varieties. For each component, values not sharing common letters in the data labels differ significantly from each other (P < 0.05) (11).

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The content and composition of acids, sugars, and sugar alcohols are important quality factors affecting directly the flavor and acceptability of the fruits and berries (*17*). Fruit acids content may also influence the stability of phenolic compounds in the fruits and the health promotion effects of the berries (*18*). The high variation found in the sugar content and the sugar/acid ratio indicates considerable difference in the sensory properties and physiological effects among the cultivars and species analyzed.

Two cultivars of *C. pinnatifida* var. *major* (Jiangou 2 and Shen78201) may have the best sensory properties and are most suitable for food industry due to the high S/A ratios. Most cultivars of *C. brettschneideri* and *C. pinnatifida* var. *major* had S/A ratio between 6–9 (Dajinxing, Quijinxing, Zizhenzhu, Caihong, Hongroushanzha, Jifu 1, Jifu 3, Xinghong 2 and Zufu 1). Those cultivars may also have good sensory profiles. All 3 forms of *C. pinnatifida* had quite low S/A ratio and total sugar contents. This suggest that fruits of this species have less pleasant tastes. The acid-rich cultivars, e.g. 947 and 8321 of *C. pinnatifida* var. *major* may be most efficient in promoting food digestion and improving blood circulation (*18*).

Profile of Phenolic Compounds

Figure 3 presents the HPLC-DAD chromatogram of the raw extract of hawthorn fruit (*C. pinnatifida* var. *major*) prepared with 80% ethanol, recorded at 280 nm (*12*, *13*). The major phenolic compounds in the extract were epicatechin, oligomeric procyanidins, glycosides of oligomeric procyanidins, phenolic acids, flavonol glycosides, and an anthocyanin. Figure 4 presents the structures of some phenolic compounds identified in the hawthorn extract.

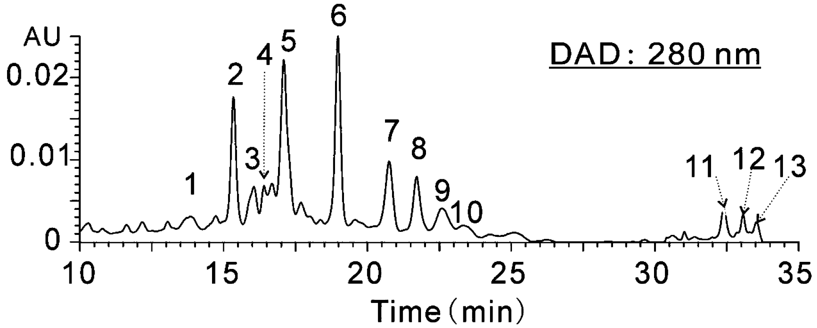

Figure 3. HPLC-DAD chromatogram of 80% ethanolic extract of fruits of the cultivar Mopan (C. pinnatifida var. major) (12, 13). Peaks: 1, ideain (14.01 min); 2, chlorogenic acid (15.33 min); 3, PA dimer-hexoside (16.03 min); 4, PA trimer I (16.43 min); 5, PA dimer I (PA B2) (17.08 min); 6, epicatechin (18.98 min); 7, PA trimer II (20.75 min); 8, unknown PA derivative (21.72 min); 9, PA tetramer (22.60 min); 10, PA trimer III (23.40 min); 11, PA dimer II (32.40 min); 12, hyperoside (33.07 min); 13, isoquercitrin (33.61 min).

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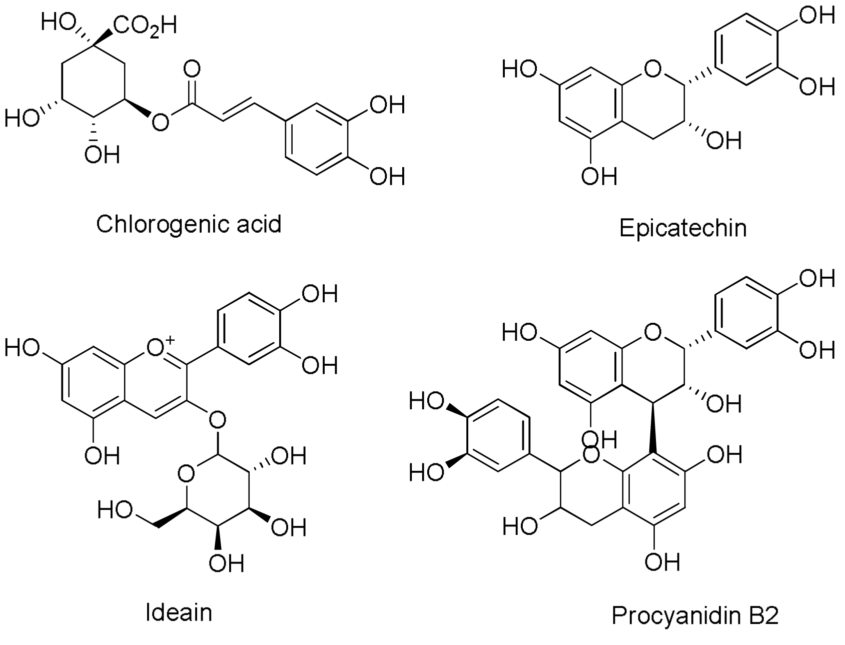
Flavonols, Chlorogenic Acid, and Ideain

Two major flavonol glycosides, hyperoside (quercetin-3-*O*-galactoside) and isoquercitrin (quercetin-3-*O*-glucoside), chlorogenic acid, and ideain were identified based on UV spectra, mass spectra and reference compounds. In addition, two flavonol glycosides were tentatively identified as quercetin-di-(methylpento)-hexoside and quercetin-methylpento-hexoside based on the UV- and mass spectra.

The content of chlorogenic acid varied in the range of 0.26-1.57 mg/g dry mass. The level of chlorogenic acid was highest in the cultivars of *C. pinnatifida* var. *major* (1.14 ± 0.28 mg/g dry mass), followed by the natural forms of *C. pinnatifida* (0.51 ± 0.16 mg/g dry mass). The lowest level of chlorogenic acid was found in the natural form of *C. scabrifolia* (0.26 mg/g dry mass) (Figure 5). Ideain was the only anthocyanin found in the hawthorn fruits. The culitvars of *C. brettschneideri* was richest in ideain. The fruits if *C. pinnatifida* contained less ideain than those of *C. brettschneideri* ($P < 0.05$) (*13*).


Chlorogenic acid

Epicatechin

Ideain

Procyanidin B2

Figure 4. Structures of chlorogenic acid, ideain, epicatechin, and procyanidin B2.

Ideain was not detected in Yun'nan shanzha (*C. scabrifolia*) at all (Figure 5). The levels of isoquercitrin were rather close among the samples (0.17-0.25 mg/g dry mass), whereas that of hyperoside varied considerably among the four groups. The cultivars of *C. brettschneideri* (0.53 ± 0.21 mg/g dry mass) and the natural forms of *C. pinnatifida* (0.42 ± 0.02 mg/g dry mass) were richer in hyperoside than the cultivars of *C. pinnatifida* var. *major* (0.26 ± 0.12 mg/g dry mass) and the natural form of *C. scabrifolia* (0.35 ± 0.06 mg/g dry mass) ($P < 0.05$, Figure 5).

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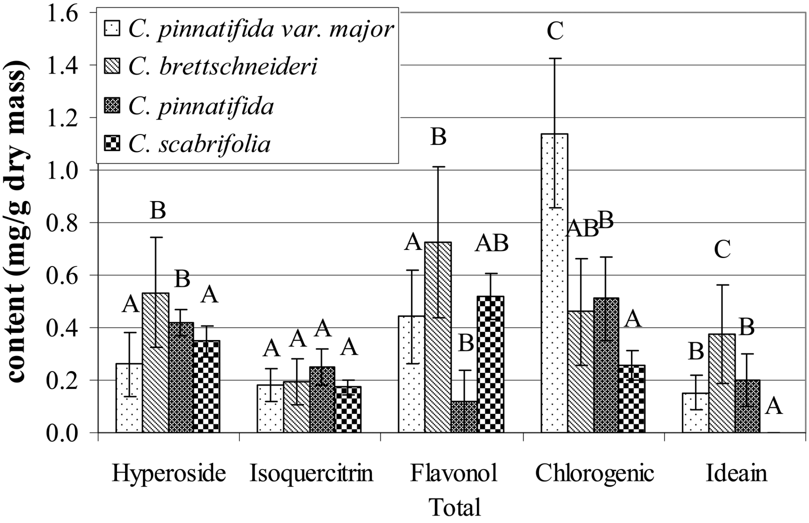

Figure 5. Content of flavonols, chlorogenic acid, and ideain in hawthorn samples of different species and varieties (13). For each component, values not sharing common letters in the data labels differ significantly from each other (P < 0.05)

Flavanols and Proanthocyanidins

Epicatechin and procyanidin B2 [epicatechin-(4β→8)-epicatechin, PA B2] were identified by comparing the retention times, UV- and mass spectra of the sample peaks with those of the reference compounds. Thirty six compounds were tentatively identified as B-type procyanidins and B-type procyanidin glycosides with polymerization index (PI) of 2-6 based on the UV- and mass spectra (*12*). To our best knowledge, this is the first report of the presence of procyanidin glycosides in hawthorn fruits.

Eight most prominent compounds were quantified using single ion recording function of HPLC-ESI-MS. The $[M+H]^+$ ions of the compounds were recorded and the peaks areas of the selected ions were used for quantification.

Epicatechin was the major flavanol and PA B2 the most abundant procyanidin (Figure 6) (*13*). The two compounds were present at roughly equal levels (0.87-12.36 mg/g dry mass). In addition to PA B2, another PA dimer (PA dimer II), a PA dimer glycoside, and three PA trimers were quantified. The content of PA dimer II varied in the range of 0.09-1.19 mg/g dry mass in the hawthorn samples analysed. The PA dimer glycoside was present at levels of 0.00-1.08 mg/g dry mass. The three PA trimers were present at levels of 0.11-2.66, 0.73-6.90, 0.01-1.24 mg/g dry mass, respectively. The contents of these compounds were higher in *C. pinnatifida* var. *major* and in *C. scabrifolia* than in the cultivars of *C. brettschneideri* and the natural forms of *C. pinnatifida* ($P < 0.05$) (Figure 6). Strong positive correlation was recognized between the content of the flavanol monomer epicatechin and and the levels of procyanidin dimers and trimers ($r^2 = 0.86 – 0.95$, $P < 0.001$) (*13*).

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Accurate quantification of proanthocyanidin is often challenging due to the difficulty in identification of unknown compounds as well as the inavailability of reference compounds. For an unknown sample, the identification and quantification of epicatechin can often be achieved without much complications. The strong correlation between the content of epicatechin and those of the dimeric, trimeric and oligomeric procyanidins could be used for a fast estimation of procyanidin content based on the content of epicatechin in hawthorn samples.

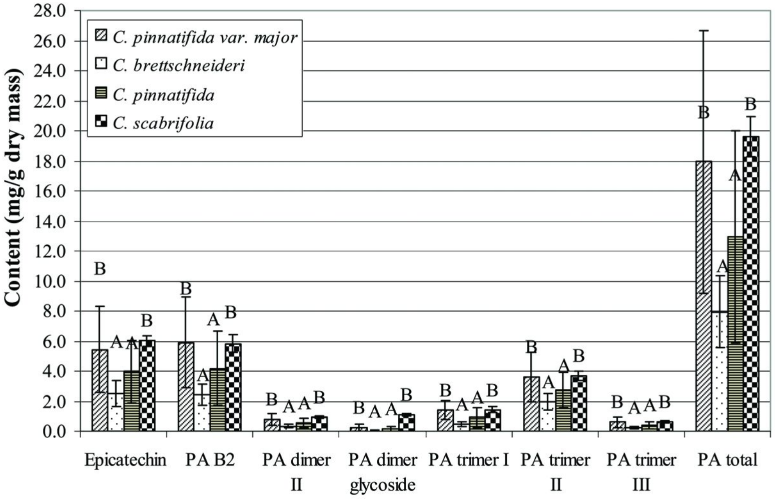

Figure 6. Content of flavanol and procyanidins in hawthorn samples of different species and varieties. For each component, values not sharing common letters in the data labels differ significantly from each other (P < 0.05) (13).

Oligomeric PAs in a European hawthorn species *C. laevigata* fruits were quantified by a combination of solid phase fractionation and HPLC analysis. The total content of PA dimers and trimers in the fruits was 0.6 mg/g DM (*19*). The total content of PAs reported in the fruits of two other European hawthorn species (*C. monogyna* and *C. oxyacantha*) varied from 14 to 26 mg/g DM (*20*). Our results showed that the levels of PAs in Chinese hawthorn fruits were higher than that reported in *C. laevigata* and close to the levels found in *C. monogyna* and *C. oxyacantha*.

Conclusions

Fructose and glucose were the major sugars in all the samples, whereas sucrose was present only in some samples. In all the samples analyzed, sorbitol was abundant, and *myo*-inositol was present. Overall, the cultivars of *C. p.* var. *major* and *C. brettschneideri* contained higher levels of sugars and higher sugar/acid ratio than the natural forms of *C. pinnatifida* and *C. scabrifolia.*

Citric acid, quinic acid, malic acid were the major fruit acids in the hawthorn fruits analyzed, citric acid being the most abundant in the native forms of *C. pinnatifida* and the cultivars of *C. p.* var. *major* and *C. brettschneideri* and quinic acid in the natural form of *C. scabrifolia.* The total acid content was highest in the natural form of *C. scabrifolia* and lowest in the cultivars of *C. brettschneideri.*

Cultivars of *C. brettschneideri* and the natural forms of *C. pinnatifida* were richer in flavonols but poorer in procyanidins than those of *C. scabrifolia* and *C. pinnatifida* var. *major.* The highest content of chlorogenic acid was found in the cultivars of *C. pinnatifida* var. *major*, and that of ideain in *C. brettschneideri.*

The high compositional variation in the fruits among different species and cultivars of hawthorn suggests the importance of raw material selection for targeted physiological effects and proper sensory properties in industrial applications of the fruits.

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Chapter 16

Bioactive Compounds from Okra Seeds: Potential Inhibitors of Advanced Glycation End Products

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Advanced Glycation End Products (AGEs) have been associated with the micro-vascular complications in diabetes and other age-related neurodegenerative diseases. We have investigated the effect of glycosylation of bovine serum albumin (BSA) in the presence of okra seed extracts. The degree of protein glycation with glucose was assessed by tryptophan AGE, AGE-induced cross-linking by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and NanoDrop spectrophotometry. Fluorescence spectra (excitation at 360 nm and read at 460nm) of BSA solution incubated for 90 days with okra seed extracts showed significant inhibitory potential (45-50%) at 0.1mg/ml concentration in a dose dependent manner. Intensity of fluorescence spectra combined with densitometry measurements exhibited 50% inhibition of glycation of BSA. We propose that the fluorescence emission spectra were altered by glycation when incubated with okra seed extracts and thus inhibited the advanced glycation end products. Further studies are needed to understand the bioactive compounds present in okra seed extracts in *in vivo* models.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Introduction

Recent studies by Huang et al. (*1*) have predicted that people inflicted with diabetes will double and the treatment costs will triple in the next 25 years. This study further highlighted and reinforced the importance of public health measures to educate and bring awareness in people about this epidemic.

The number of people becoming obese in United States and also in India has been rising steadily for many years (*2*). The data shows that one in five people with Type-2 diabetes are morbidly obese while 1 in 3 African-American adults with diabetes, are morbidly obese or approximately 100 pounds overweight (*1–3*). The major cause and control of Type-2 diabetes is not clear but recent studies have focused on dietary factors which may be involved in the regulation and control of this devastating disease.

The accumulation of Advanced AGEs due to non enzymatic glycation of proteins has been implicated in both Type 2- diabetes and cardiovascular disease (*4–6*). This reaction takes place between reducing sugar and the free amino group of the protein, ε-amino group of lysine and the guanidine group of arginine forming an Amadori product) (*7–9*). The formation of complex glycoxidation products, carboxylated methyl lysines (CMLys), brown pigments, protein cross-links, antigenic AGEs and pentosidine have been shown to accumulate irreversibly in several human and animal tissues during aging (*10*, *11*). This process the so called the Maillard reaction or advanced glycation occurs slowly *in vivo* causing damage to proteins. Further studies have illustrated the role of AGEs in the development of several pathophysiological associated age-related neurodegenerative diseases such as Alzheimer's, arthritis, end-stage renal disease, nephropathy and neuropathy and cataract formation (*10–16*). Oxygen radicals are implicated in Maillard reaction damage to proteins (*14*).

Since obesity is a major environmental risk factor for diabetes (*1–3*), lifestyle changes have been recommended to reduce obesity. Low-fat low-calorie vegetarian fruit diet rich in polyphenols and antioxidants may be helpful in the prevention and treatment of various age-related neurodegenerative diseases. Studies have indicated that a high fat diet supplemented with flavonoids may reverse the progression of diabetes, heart disease and cancer (*17–22*). In the present study okra seed (Fig. 1) extracts were examined to see their beneficial inhibitory effects on AGEs formation and antioxidant activity.

Experimental Procedures

Microwave Extraction

A rapid microwave-assisted enhancement reaction chemistry procedure (*23–27*) was utilized for the extraction of flavonol glycosides using methanol or ethanol solvents. Briefly, 5g of powered okra seeds was extracted with methanol (5ml) in the microwave oven (MW) at low power setting. After filtration each extract was evaporated under nitrogen to dryness and used for chemical analysis and bioassays.

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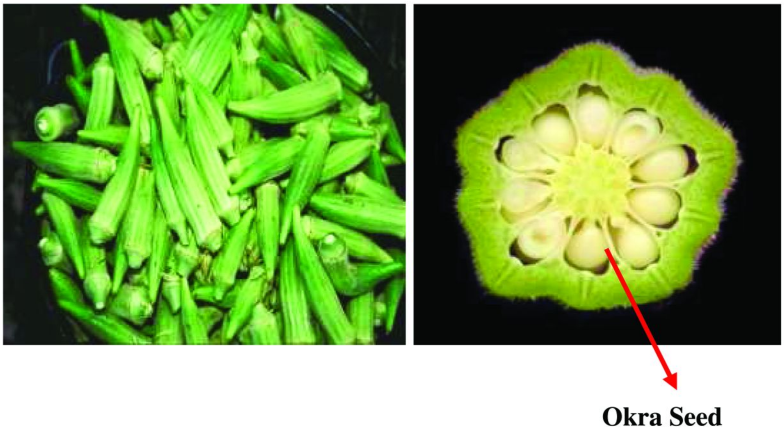

Figure 1. Okra: Abelmoschus esculentus (L.) Moench.

Determination of Total Phenolics and DPPH Radical Scavenging Activity

Assays were performed with methanolic extracts. Phenolics were measured using the Folin- Ciocalteau reagent and expressed as mg cholorogenic acid equivalent per gram wet weight. Anti-oxidant activity was determined using a 2, 2-diphenyl-1-picrylhydrazyl assay and is expressed as mg trolox equivalent per gram wet weight (*34*).

Identification of Bioactives by LC-MS/MS

Flavonoids with different sugar moieties (Fig. 2) were identified by high-performance liquid chromatography coupled with mass spectrometry (HPLC–MS–MS) (*28*, *29*). The HPLC system (Shimadzu Co., 10VP Series, Columbia, MD, USA) employed a HypersilGold C_{18} (3 µm particle size; 150 mm length × 3.0 mm ID; Thermo Electron Co., Bellefonte, PA). Five microlitre was injected onto the column and a gradient elution was used for separations. Solvent A consisted of 10% MeOH in H_2O adjusted to pH 3.5 with formic acid. Solvent B consisted of 20% H_2O (pH 3.5), 20% MeOH, and 60% acetonitrile. At a flow rate of 0.3 mL min^{-1}, the following gradient was used: 0 min, 100% A; 10 min 20% A; 20 min, 40% A; 40 min, 0 % A; held at 0% A for 15 min. Five minutes of equilibration at 100% A was performed before and after each injection. Effluent from the column was introduced into a triple-quadrupole mass spectrometer (Micromass Inc., Beverly, MA, USA) equipped with a pneumatically-assisted electrospray ionization source (ESI). Mass spectra were acquired in the negative ion mode under the following parameters: capillary voltage, 3 kV; source block temperature, 120 °C; desolvation gas temperature, 400 °C. Nitrogen was used as the drying and nebulizing gas at flow rates of approximately 50 and 450 L/h. For full-scan HPLC–ESI-MS analysis, spectra were scanned in the range of 50–1200 *m/z*. Data acquisition and processing were performed using a Mass-Lynx NT 3.5 data system (Micromass Inc., Beverly, MA, USA).

NMR Spectroscopy of Okra Seed Extract

One- Dimensional Proton, ^{13}C NMR spectra were recorded on a Bruker 500 and 125 MHz respectively at room temperature using 3 mm tubes. Samples (3 mg) were dissolved in CD_3OD. Attached-Proton-Test (APT) spectra were recorded to identify "multiplicity" (quaternary, CH, CH_2 or CH_3) of peaks in a ^{13}C spectrum as described previously (*30*, *31*).

Glycation of BSA

BSA (0.1g/ml), was incubated at 37 °C with 1M glucose in 0.4M phosphate buffer, pH 7.4 containing 0.02% sodium azide to prevent degradation (*8*, *13*). After thorough mixing okra seed extract (0.1g/ml) was added. A corresponding control without the okra seed extract was also prepared and the mixture kept in the same incubation chamber for a period of 30-90 days.

SDS-PAGE Analysis

Effect of Okra seed extracts on glucose-induced glycation of BSA in the absence and presence of aqueous okra seed extracts was resolved and analyzed via SDS-PAGE analysis (Fig. 4). Soluble protein and molecular weight standards (kDa) were loaded onto 8% polyacrylamide gel and were visualized via Commassie blue staining procedure.

Densitometry Measurements

Densitometry plots were generated with Image J software of the sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) of glycated BSA with and without okra seed extract. The sample concentrations (1A,1B and1C, 0.1mg/ml) without okra seed and (2A, 2B and -2C, 0.1mg/ml) with okra seed extract treated BSA samples are shown in (Fig. 5).

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Advanced Glycation End Products (AGE) Analysis

Fluorescence of the samples was measured at the excitation and emission maxima of 360 and 460 nm, respectively (*13*). An un-incubated blank containing BSA, glucose and okra seed extract as an inhibitor was also prepared and measured (Fig. 6).

Protein Concentration via NanoDrop Spectrophotomter and Fluorospectrometer

The individual variation in BSA concentrations in the presence and absence of okra seed extracts were determined utilizing Nano Drop spectrophotometer and fluorospectrometer which has the ability to analyze multiple emission profiles from a single sample (Fig. 7).

Results and Discussion

The okra seeds (5 g) were treated with methanol (5ml) and heated using a microwave oven. The extracts/aliquots were used for the identification of major flavonoids as described in the experimental section. Results of liquid chromatography-mass spectrometry data as presented in Table 1 revealed five major flavonoids. A representative LC-MS spectra of quercetin-diglucoside is described in Fig. 3. From the analysis of the LC-MS/MS data in the negative ion mode five flavonoids exhibited fragment ions $[M-162]^+$ after the loss of the respective sugar moiety (glucose, rhamnose or galactose) for glycosides and $[M-132]^+$ for arabinosides (Fig. 2). The LC-MS/MS data presented in Table 1 is based on LC-MS mass fragmentation pattern and also comparison with authentic standards.

Table 1. Identified Flavonoids from Okra Using LC-MS-MS

$[M-H]^-$	*Name of The Compound*	*Retention Time (min)*
433	3,5,7,3′,4′-pentahydroxyflavonol-3-O-α-L-arabinofuranoside	22.27
447	Kaempferol-3-O-glucoside	25.76
609	Quercetin-3-O-rutinoside	20.02
463	Quercetin-3-O-glucoside	20.95
625	Quercetin-3-O-diglucoside	16.76

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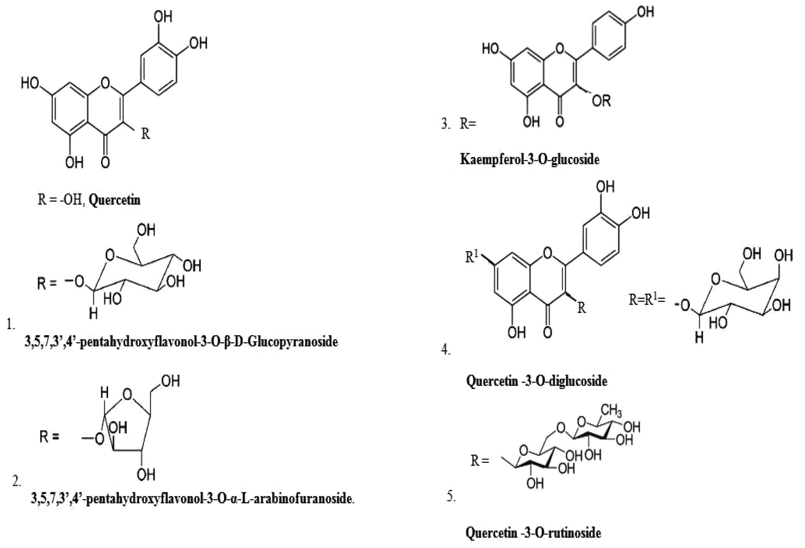
R = -OH, Quercetin
1.
3,5,7,3',4'-pentahydroxyflavonol-3-O-β-D-Glucopyranoside
2.
3,5,7,3',4'-pentahydroxyflavonol-3-O-α-L-arabinofuranoside.
3. R=
Kaempferol-3-O-glucoside
4.
R=R1=
Quercetin -3-O-diglucoside
5.
R=
Quercetin -3-O-rutinoside

Figure 2. Chemical structures of flavonoids identified from okra seed extract.

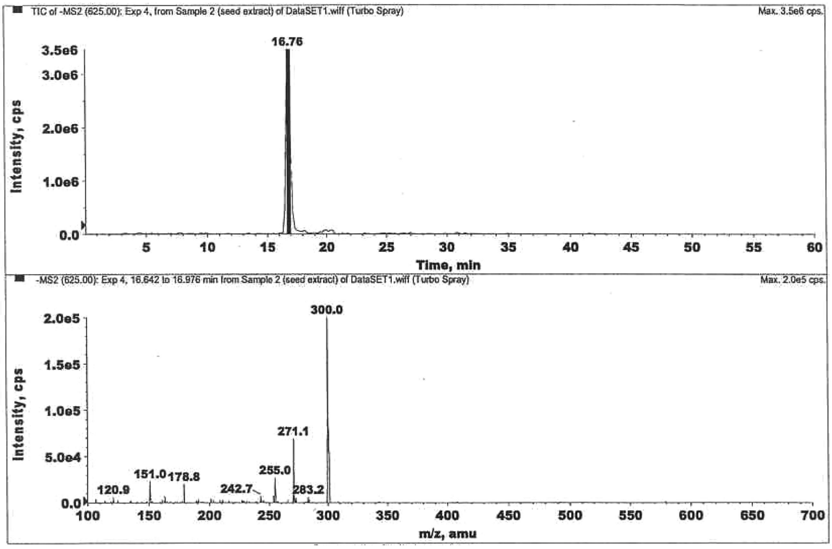

Figure 3. Typical HPLC analysis top panel (Rt=16.76 min) and Electrospray-ionization mass spectra of quercetin-3-O-diglucoside bottom panel illustrates [M-H]-= 625 based on LC/MS/MS fragmentation pattern in the negative ion mode. As illustrated in Figure 2, the compounds present in okra seed extract were flavonoids with different sugar moieties. Their structural identity was determined using ESI-MS.

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Discussion

The okra (*Abelmoschus esculentus*) vegetable is a valuable source of micronutrients (*33*) and is a major source of flavonoids (Fig. 2). Polyphenolics present in okra seed have potent antioxidant properties (Table 2) and thus inhibit generation of free radicals and cell death (*33–35*).

Table 2. Distribution of Phenolics and Antioxidant Activity in Okra

Okra parts	*Phenolic (mg/g)*	*Antioxidant Activity*
Skin	0.20	0.22
Skeleton	0.09	0.23
Seed	2.85	7.55
Stem	0.13	0.59

Assays were performed with methanolic extracts. Phenolics were measured using Folin- Ciocalteau reagent and expressed as mg chlorogenic acid equivalent per gram wet weight. Anti-oxidant activity was determined using a 2, 2-diphenyl-1-picrylhydrazyl assay and is expressed as mg trolox equivalent per gram wet weight. Values are means of duplicate determinations.

Since oxidative stress is clearly associated with cellular dysfunction such as in diabetes, the bio-active compounds present in okra seed may play a key role in the prevention and treatment of diabetes. Upon administering okra juice to volunteers, its soluble fiber helped to lower their serum cholesterol and reduced blood sugar levels. It has been suggested that *In vitro* binding of bile acids with the phenolics present in okra is the highest (16%) in comparison to other vegetables (*36*). The faecal excretion of bile acids has been hypothesized as a possible mechanism by which soluble okra fiber lowers cholesterol (*36*). The mucilage or the insoluble portion helps to maintain intestinal tract healthy and may be helpful in alleviating inflammation in ulcerative colitis/colon cancer and inflammatory bowel disease syndromes .

In the dietary context, the most important compounds present in okra seeds are polyphenolic flavonols namely, quercetin and kaempferol, in addition to cinnamic acid derivatives (Fig. 2). The flavonols present in okra seeds are in glycosylated form, with one, or two, sugar moieties (hexoses, pentoses, rhamnoses) attached to flavonol hydroxyl groups (*30–32*). Further studies have shown that flavonols have anti carcinogenic and antioxidant properties (*32–37*). More recent studies have pointed out the role of such small molecules as sirtuin activators and a good target for diseases of aging such as diabetes, cancer, metabolic diseases, inflammation and neurodegenerative diseases (*38–41*).

We have investigated the effect of glycosylation of bovine serum albumin (BSA) in the presence of okra seed extracts. The glycosylated and non-glycosylated protein bands were resolved on SDS- polyacrylamide gel electrophoresis (SDS-PAGE). The degree of protein glycation with glucose was assessed by tryptophan AGE, AGE-induced cross-linking by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and NanoDrop spectrophotometry.

The Fig. 4 presents effect of okra seed extracts on glucose-induced glycation of BSA in the absence and presence of aqueous okra seed extracts by SDS-PAGE analysis. Soluble protein was loaded onto 8% polyacrylamide gel.

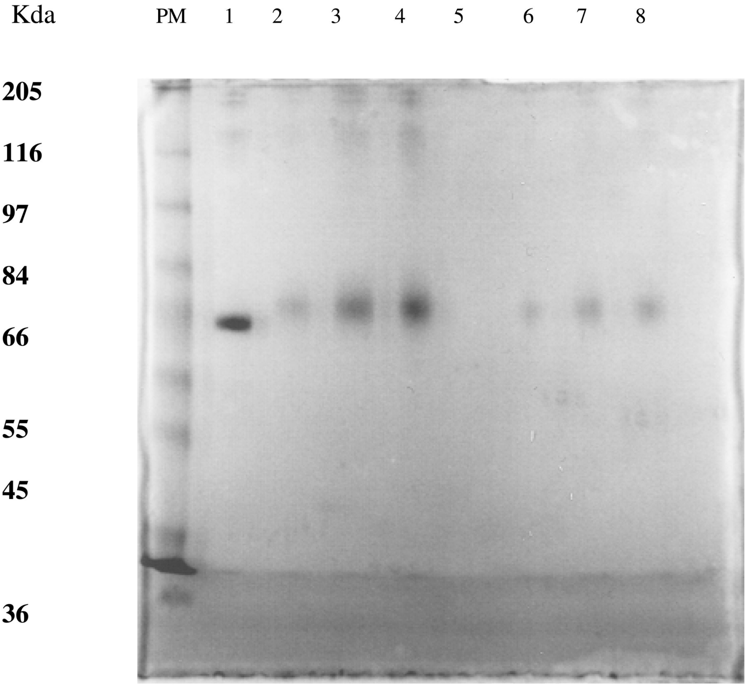

*Figure 4. *Effect of okra seed extracts on glucose-induced glycation of BSA in the absence and presence of aqueous okra seed extracts by SDS-PAGE analysis. Soluble protein was loaded onto 8% polyacrylamide gel. Molecular weight standards (kDa) are indicated alongside of the gel. Lane PM- Molecular weight markers; Lane 1- BSA standard 0.1mg/mL; Lane 2- Sample 1A; Lane 3- Sample 1B; Lane 4- Sample 1C; Lane 6- Sample 2A; Lane 7- Sample 2B; Lane 8- Sample 2C.*

The protein bands in this SDS gel were resolved and visualized by Coomassie blue staining. The positions of molecular mass markers (M) in (kilodaltons) are shown to the left of the gels in panels treated without/with okra seed extracts) The results of SDS Page combined with densitometric plots as presented in (Fig. 5) with BSA (0.1mg/ml, 1A-1C and 2A-2C) without and with okraseed extract show the inhibition of glycation and AGE. Fluorescence measurements at the

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excitation and emission of 360 and 460 nm also show the inhibition of glycation and AGE with okra seed extracts versus BSA and glucose alone when incubated for 90 days (Fig 6). This was further substantiated with protein concentration measurements via nanodrop spectrophotometer (Fig. 7) showing inhibition at different concentrations in comparison to no inhibitor added at the physiological concentrations. The degree of protein glycation as assessed by tryptophan AGE (Fig. 6), AGE-induced cross-linking by SDS-PAGE showed significant inhibitory potential (45-50%) at 0.1mg/ml concentration in a dose dependent manner. Intensity of fluorescence spectra combined with densitometry measurements exhibited 50% inhibition of glycation of BSA with glucose (Figs. 4-7).

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Glycation of BSA	Glycation of BSA with Okra seed extracts	Concentration of the Sample
1A	2A	0.1mg/mL
1B	2B	0.2mg/mL
1C	2C	0.3mg/mL

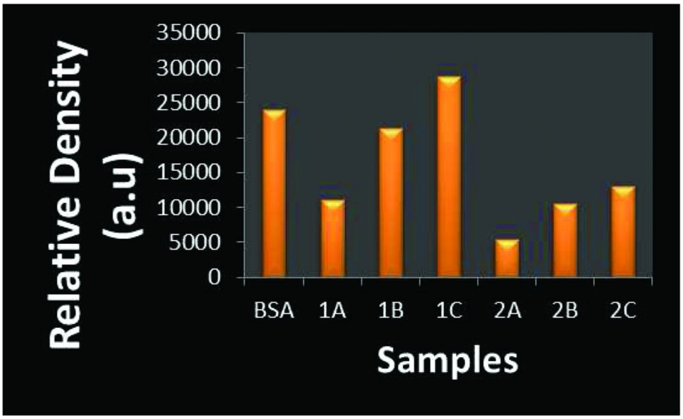

Figure 5. Densitometry plots generated with Image J software of the sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) of glycated BSA without okra seed extract (1A-1C) and (2A-2C) with okra seed extract treated samples.

We believe fluorescence emission spectra were altered upon glycation when incubated with okra seed extracts *in vitro* and thus inhibited the advanced glycation end products (*13–16*, *42–49*).The glycosylated BSA protein concentration was also decreased as a result of okra seed treatment as measured via nanodrop spectrophotometer (middle curve Fig. 7) . Glycosylation of BSA in the presence of okra seed extracts leads to a change in the conformation of the protein probably due to an increased exposure of tryptophan residues (*42–48*). Thus the glycation of BSA in the absence and presence of aqueous okra seed extracts by SDS-PAGE analysis. Soluble protein was loaded onto 8% polyacrylamide gel. Molecular weight standards (kDa) are indicated alongside of the gel. Lane PM- Molecular weight markers; Lane 1- BSA standard 0.1mg/mL; Lane 2- Sample 1A; Lane 3- Sample 1B; Lane 4- Sample 1C; Lane 6- Sample 2A; Lane 7- Sample 2B; Lane 8- Sample 2C. bioactive compounds present in okra seed extracts may serve the same mechanisms of inhibition *in vivo* as well.

Potential Inhibition of Nonenzymatic Glycosylation of HDL Apolipoprotein A-1 Cholesterol Levels with Okra Juice

Hyperglycemia in diabetes type-2 is caused by the post-translational nonenzymatic glycosylation of plasma and cellular proteins (*49*). As illustrated in earlier the reaction of glucose with protein alpha and (ε-amino group of lysine and the guanidine group of arginine forming an Amadori product) occurs *in vivo* (*7*, *9*, *11*, *12*). This reaction leads to the loss of charged groups on lysine residues causing conformational changes in proteins. We recently elucidated HDL apolipoprotein A-1 cholesterol levels (*50*) in severely controlled Type-2 diabetes patients and found increased levels of glycosylated HDL apolipoprotein A-1 Cholesterol. We also carried out studies to measure inflammatory molecular markers of coronary risk such as high-sensitivity C-Reactive Protein (hs-CRP) and interleukin-6 (IL-6) and found that elevated levels of IL-6 accelerates the hs-CRP Pathway (*50–54*).

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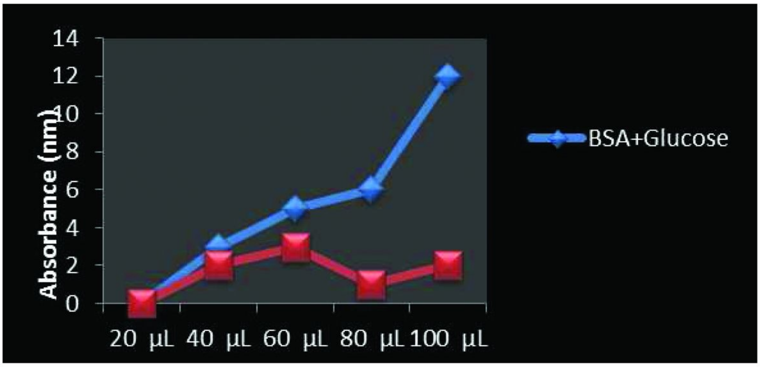

Figure 6. Fluorescence spectra exhibiting glycation of BSA with (lower curve) and without okra seed extract (upper curve).

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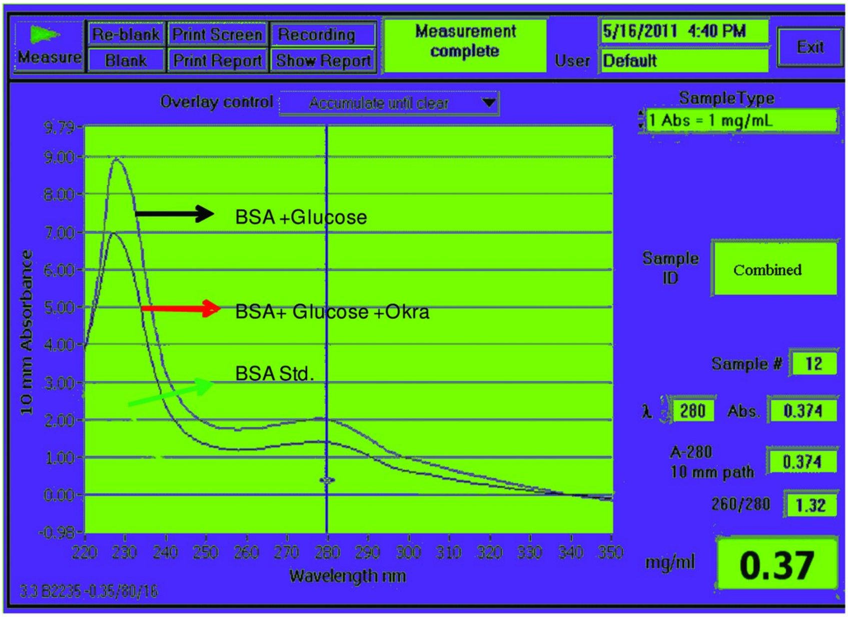
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Overlay control Accumulate until clear

SampleType 1 Abs = 1 mg/mL

Sample ID Combined

Sample # 12

λ 280 Abs. 0.374

A-280 10 mm path 0.374

260/280 1.32

mg/ml 0.37

3.3 B2235 -0.35/80/16

Figure 7. Evaluation of BSA protein concentration in the presence and absence of okra seed extract using NanoDrop Spectrophotometer.

Since in diabetes patients hyperglycemia results in the nonenzymatic glycosylation of many proteins (glycation is the attachment of sugar to proteins), a basal level of HDL apolipoprotein A-1, a cardiovascular biomarker and a major carrier protein in High Density Lipoprotein cholesterol undergoes glycation in normal individuals. But there is a nearly 400% increase in the level of HDL-apoA-1 glycation in diabetic patients (*49*). The level of glycation has been positively correlated with the degree of hyperglycemia and such a functional abnormality in HDL-apoA-1 is responsible for the accelerated development of atherosclerosis in diabetic patients (*49*). Since there is 16 % binding of bile acids with okra juice (*36*) food fractions prevent their reabsorption and stimulate plasma and liver cholesterol transformation to synthesize more bile acids thus lowering cholesterol. Since advanced glycation end products and atherogenic glycosylated or oxidized LDL is formed *in vivo in diabetic patients*, we believe the therapeutic mediation of inhibiting protein glycation by Okra juice would not only prevent the progression of diabetes but also cardiovascular disease as well. Furthermore the levels of inflammatory molecular markers of coronary risk such as high-sensitivity C-Reactive Protein (hs-CRP) and interleukin-6 (IL-6) may also be lowered (*50–55*).

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Supersized LDL and HDL Cholesterol Carriers Key to Longevity of Life and The Potential Role of Okra Juice

Potential inhibition of glycosylated or oxidized LDL by Okra seed extract may suggest an extremely valuable antidiabetic and antiatherogenic therapy.

Recent studies have shown that smaller sized LDL-cholesterol particles get stuck or clog the arteries much faster than their supersized LDL-particles in some individuals. Studies by Barzilai et al. indicated that supersized cholesterol carriers (large particles of both HDL cholesterol and LDL cholesterol) may protect against heart disease and thus lead to exceptional longevity (*56*). These studies attributed enhanced longevity with specific biological genetic factors. It is known that oxidized or glycosylated LDL is more atherogenic than native LDL and AGE and atherogenic LDL are both present *in vivo* in diabetic patients. Therefore, the inhibition of glycation and oxidation by the antioxidant polyphenolics in Okra juice may play a role in preventing oxidation as well as formation of glycosylated atherogenetic LDL. This process will then increase the size of LDL particles. By using SELDI ProteinChip MS technology as a rapid method for the identification of HDL-apoA1 and high sensitivity C-Reactive Protein (hs-CRP) in diabetic patients who have cardiovascular disease, a comparative molecular characterization and expression levels of super sized HDL and LDL can be achieved.

Further development of these techniques may facilitate both epidemiological and therapeutic trials in assessing the role of hs-CRPand HDL apolipoprotein A-1 and their glycosylated products in atherosclerosis.

Our long term goal is to find a natural product which can therapeutically prevent protein glycation and stop Amadori Products (AP) in the initial stages of advanved glycation end products. Although a well-studied AGEs inhibitor aminoguanidine reacts with AP and stops the progression of diabetic complications but an inexpensive and non-toxic natural product would be ideal for the prevention and treatment of diabetic complications. Future studies are in progress to evaluate the inhibitory nature of bioactives present in okra seeds to prevent the oxidation and glycosylation of LDL, a cardiovascular biomarker.

A recent Iranian publication Grasas Y Aceities, (*57*) reported that the methanol extract of okra seeds had a remarkable antihypoxic effect in both models of circulatory and haemic hypoxia. The seed extract had protective effect against hypoxia-induced lethality in mice. The present studies presented have highlighted the potential benefits and importance of highly antioxidant nature of flavonoids present in Okra Seeds. Further okra research along these lines may have potential implications on diabetes and cardiovascular biomarkers such as HDL-apoA1, hs-CRP, Il-6 protein expression profiles and blood-brain barrier chemistry.

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Chapter 17

Jasmonates: Plant Stress Hormones as Anticancer Agents

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Jasmonates are a group of plant stress hormones which are among the most potent regulators of defense-related mechanisms in plants. In recent years, several groups have reported that jasmonates are endowed with anti-cancer activities both in vitro and in vivo. Jasmonates were shown to induce death and inhibit the proliferation and the migration of various cancer cell types, including drug-resistant cells. Moreover, jasmonates were shown to impair the angiogenic process, which is essential for tumor progression. In line with their anti-cancer activities in vitro, jasmonates were shown to increase the survival of lymphoma-bearing mice and to inhibit the development of lung metastases in a mouse melanoma model. Importantly, jasmonates are highly selective towards cancer cells and have little or no effect on normal cells, creating a wide therapeutic window. Recently, a first-in-man study demonstrated that methyl jasmonate has a beneficial effect in treating human pre-cancerous and cancerous skin lesions. Several mechanisms were shown to mediate the anti-cancer activities of jasmonates. These include: direct perturbation of mitochondria, production of reactive oxygen species, induction of cellular differentiation, inhibition of aldo-keto reductases, upregulation of several pro-apoptotic proteins and downregulation of anti-apoptotic proteins. A number of research groups have taken the natural jasmonate compounds as a starting point to prepare and evaluate a wide variety of synthetic jasmonate derivatives. Several of these

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derivatives exhibited enhanced anti-cancer activities in vitro and in vivo. While the vast majority of studies on jasmonates as potential drugs have been performed in the cancer arena, these compounds have also been evaluated as anti-parasitic and anti-inflammatory agents. In conclusion, jasmonates present a unique class of anti-cancer compounds which deserves continued research at the basic, pharmaceutical and clinical levels in order to yield novel chemotherapeutic agents against a range of neoplastic diseases.

The Anticancer Activity of Jasmonates

Cancer is a leading cause of death worldwide (*1*). According to the World Health Organization (WHO) cancer is accountable for 7.6 million deaths each year. Despite extensive developments in the fields of oncology and translational research, the discovery of novel treatments that substantially improve the survival of cancer patients remains frustratingly low (*2*). Two major obstacles for successful cancer treatment are the resistance of cancer cells to existing chemotherapeutics and the high level of toxicity induced by many chemotherapeutic drugs (*1*). As the primary tumor can often be surgically removed, the main challenge of adjuvant therapy is to eliminate the spread of metastases and prevent their development in vital organs. Indeed, the leading cause of cancer mortality is the development of metastases that are resistant to conventional chemotherapy (*3*). Therefore novel anti-cancer agents that may overcome drug-resistance and inhibit the spread of metastases while exhibiting high selectivity towards cancer cells are in constant demand. The jasmonate group of plant stress hormones fulfills all of these criteria and holds great promise as a potential source of anti-cancer agents.

Jasmonates are a group of plant stress hormones which are among the most potent regulators of defense-related mechanisms in plants. Naturally occurring jamonates include numerous compounds the most prominent of which are jasmonic acid (JA, Figure 1) and its methyl ester, methyl jasmonate (MJ, Figure 1) (*4*). Jasmonates are induced in plants in response to various types of stresses and mediate the defense response to mechanical and infectious insults. Jasmonates regulate plant gene expression including defense related genes and induce the production of various phytochemicals. These phytochemicals can interact directly with plant invaders, such as herbivores, bacteria and fungi, to bring about their neutralization. Although a considerable body of knowledge has been accumulated regarding jasmonate-regulated gene expression, relatively little is known about the signaling pathways mediating these actions of jasmonates. Interestingly, jasmonates can also regulate gene expression in herbivorous insects, suggesting that jasmonate signaling pathways may be conserved between plants and animals. In addition to their role in defense-related mechanisms, jasmonates also regulate additional functions in plant physiology including growth, development and reproduction (*4*). As a part of their role in plant development and defense, jasmonates were shown to induce programmed cell death in plant cells (*5*).

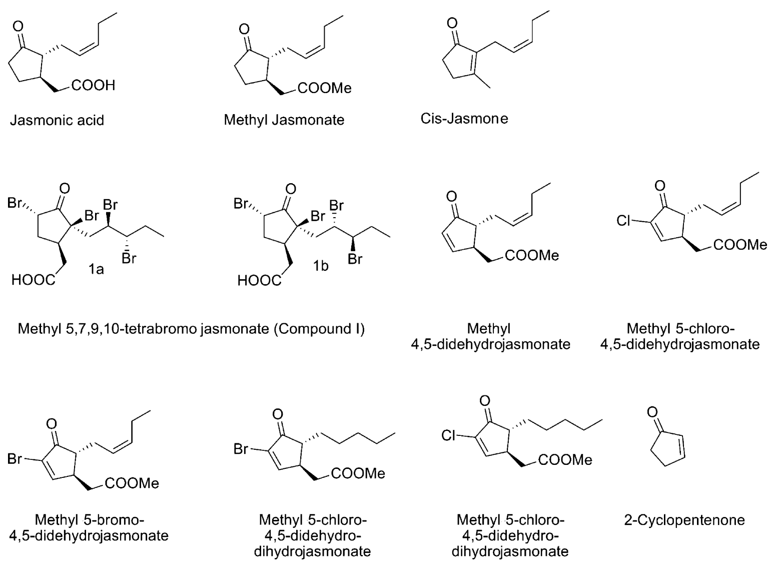
Jasmonic acid
Methyl Jasmonate
Cis-Jasmone
1a
1b
Methyl 5,7,9,10-tetrabromo jasmonate (Compound I)
Methyl 4,5-didehydrojasmonate
Methyl 5-chloro-4,5-didehydrojasmonate
Methyl 5-bromo-4,5-didehydrojasmonate
Methyl 5-chloro-4,5-didehydro-dihydrojasmonate
Methyl 5-chloro-4,5-didehydro-dihydrojasmonate
2-Cyclopentenone

Figure 1. Chemical structures of natural and synthetic jasmonates.

Originally found as major constituents in the etheric oil of jasmine, jasmonates are expressed throughout the plant kingdom (*4*). However, several plants express relatively high levels of jasmonates. These include: jasminum, olive, ginger, rosemary, honeysuckle and more (*6*). Interestingly, several studies have demonstrated the anti-cancer activities of jasmonate-rich plants (for review see (*6*)).

Structurally and biosynthetically, jasmonates are cyclopentanones that belong to the family of oxygenated fatty acid derivatives, collectively called oxylipins, which are produced via the oxidative metabolism of polyunsaturated fatty acids. Jasmonate biosynthesis in plants is analogous to eicosanoid biosynthesis in animal cells. Animal eicosanoinds (C20) are lipid bioregulators that are synthesized from arachidonic acid and function as regulators of cell immune response, differentiation and homeostasis. In plants, jasmonates are derived from C18 α-linolenic acid (18:3) and control similar activities (*4*). Indeed, the structure of jasmonates resembles that of certain prostaglandins (*7*).

Several groups have reported in recent years that jasmonates and some of their synthetic analogs, exhibit anti-cancer activity in vitro and in vivo. Our initial report, published in 2002 (*8*), revealed that JA inhibited the proliferation of several human cancer cell lines including breast, prostate, and melanoma cells and induced death in human lymphoblastic leukemia cells. MJ induced death in various human and mouse cancer cell lines including: breast, prostate, melanoma, lymphoma and lymphoblastic leukemia cells (*8*). Subsequent reports demonstrated that jasmonates can induce death or inhibit the proliferation of various other cancer cell lines including lung carcinoma (*9–11*), colon carcinoma (*10*), melanoma (*12*), cervical carcinoma (*13*), sarcoma (*14*), glioma (*15*), myeloid leukemia (*16–18*) and neuroblastoma (*19, 20*) cells. Furthermore jasmonates increased the life span of T-cell lymphoma-bearing mice, thus demonstrating an anti-cancer effect in vivo (*8*). Recently, a first-in-man study demonstrated that MJ has a beneficial effect in treating human pre-cancerous and cancerous skin lesions (*21*). As most chemotherapeutic drugs are designed to induce cell death, toxicity to normal tissue is difficult to avoid during treatment (*1*). Thus, selectivity towards cancer cells is a highly desirable characteristic for novel anti-cancer agents. Jasmonates induce death selectively in cancer cells while non-transformed cells are relatively unaffected by jasmonate treatment. While MJ at 1mM induced death in human lymphoblastic leukemia cells, normal lymphocyes remained relatively unaffected (*8*). In another study, MJ at 3 mM decreased the viability of four different cervical cancer cell lines by over 50% while normal foreskin primary keratinocytes were significantly more resistant to MJ treatment, with reduction of only ~15% in cell viability (*13*). Finally, an ultimate experiment that demonstrated the selectivity of MJ was performed on blood samples obtained from chronic lymphocytic leukemia (CLL) patients and contained a mixed population of normal and leukemic lymphocytes. MJ selectively killed the leukemic cells in the samples, gradually decreasing the percentage of leukemic cells and increasing the percentage of normal lymphocytes, in a dose dependent manner (*22*). In line with these results, a positive correlation was found between the percentage of leukemic cells in blood samples of CLL patients and the level of cytotoxicity induced by MJ (*23*).

Most cancer-related deaths are not attributed to the development of the primary tumor, which can often be surgically removed, but rather to the dissemination of metastases and their development in vital organs (*3*). Therefore, agents that may block the spread and development of metastases may prove to be potent anti-cancer agents. Cell migration is an essential component of the metastatic process (*24*). The ability of MJ to impair cell migration was evaluated in B16-F10 mouse metastatic melanoma cell line. MJ, at non-cytotoxic concentrations, inhibited the migration of the cells in a dose-dependent manner (*12*). Moreover, MJ exhibited an anti-metastatic effect in vivo, inhibiting the development of melanoma metastases in the lungs of mice (*12*). These results demonstrate the ability of MJ to interfere with the metastatic process, plausibly by inhibiting cell migration.

Another cause of cancer related mortality is the failure of treatment due to drug resistance. Since drug resistance plays a crucial role in the clinical outcome of cancer treatment (*1*), it was interesting to evaluate the effect of jasmonates on drug-resistant cancer cells. Two different mechanisms of drug-resistance were studied: p53-mutation (*25*) and P-glycoprotein over-expression (*12*).

p53 is a tumor suppressor gene. Its tumor-suppressive activity involves the induction of cell cycle arrest or apoptosis (a form of programmed cell death which will be further elucidated in the next section). Mutation in p53 occurs in more than 50% of human cancers (*26*). Various tumors consisting of mutant p53-expressing cells exhibit high resistance to radiation and chemotherapeutic drugs. Circumventing this abnormal resistance is a major challenge in cancer therapy (*27*). The effect of jasmonates on drug-resistant cells harboring mutated p53 was evaluated using two clones of B-lymphoma cells. One clone expressed wild-type p53 and the other expressed mutated p53. While the mutant p53-expressing cells where resistant to the radio-mimetic neocazinostatin (NCS) and to the chemotherapeutic agent bleomycin, compared to their wild-type p53-expressing counterparts, they did not exhibit resistance to jasmonate treatment. JA and MJ were both equally cytotoxic to both clones (*25*). In another study, the cytotoxicity of MJ was evaluated in several cervical carcinoma cell lines expressing either wild-type or mutant p53. MJ induced death in the different cervical carcinoma cell lines regardless of their p53 status (*13*). Thus, jasmonates can overcome drug resistance induced by p53-mutations.

Another model of drug resistance is the P-glycoprotein over-expression mechanism. P-glycoprotein is an efflux pump that is normally expressed in intestine, kidney and liver tissues as well as in endothelial cells of the blood-brain barrier. It protects normal tissue by excreting various cytotoxins out of the cells. P-glycoproein is highly expressed in many tumor types. It excretes many chemically unrelated drugs out of the cancer cell endowing the cell with a multi-drug resistant phenotype. Drugs that are P-glycoprotein substrates include taxanes, anthracyclins, vinca alakaloids and epipodophylotoxins (*28*). The effects of jasmonates were evaluated on two B16-F10 melanoma clones, one expressing low levels of P-glycoprotein and the other expressing high levels of P-glycoprotein (*12*). While the high P-glycoprotein expressing clone exhibited resistance to the chemotherapeutics doxorubicin, vinblastine and colchicine, both clones were equally sensitive to the cytotoxic activity of jasmonates (*12*). Inhibition of P-glycoprotein with a selective inhibitor sensitized the resistant cells to chemotherapeutic agents but did not affect their sensitivity to jasmonates (Flescher, personal communication). Furthermore, the high P-glycoprotein expressing melanoma cells were also shown to be highly metastatic and exhibit increased spread to the lungs as well as higher cell motility rates (*29*). In addition to its ability to kill these melanoma cells, MJ, at non cytotoxic concentrations, inhibited the migration of these highly metastatic and drug-resistant cells (*12*). These results indicate that unlike many conventional chemotherapeutic agents, jasmonates are not substrates of the P-glycoprotein efflux pump and thus have the potential of eliminating drug-resistant cancer cells.

In a recently published article, Pereira-Lopes et al. (*30*) demonstrated that not only do jasmonates directly affect tumor cells, they may also have an effect on the development of tumor vasculature. Angiogenesis, the formation of new capillaries from pre-existing vessels, is a crucial step in tumor progression and is therefore an attractive target for therapeutic intervention (*31*). Pereira-Lopes et al. discovered that MJ impaired vascular growth in a Chorioallantoic Membrane of the Chicken Embryo (CAM) model of angiogenesis. The effect of MJ seemed to depend on the applied dosage. While, millimolar concentrations of MJ significantly reduced capillary growth, an effect which is most likely associated with direct cytotoxicity of MJ towards endothelial cells, lower concentrations (<100 μM MJ) typically resulted in disordered vascular growth, forming many of capillary anastomoses and dysfunctional vessels. Thus, the anti-cancer activity induced by MJ in vivo might result from its combined effect on tumor cells and tumor vasculature.

Almost all curative chemotherapy regimens for cancer employ multi-agent drug combinations (*32*). Several studies have evaluated the potential of combining MJ with different cytotoxic and chemotherapeutic agents, with the hope of identifying combinations with enhanced cytotoxic effects, compared to that of each agent by itself. Four conventional chemotherapeutic drugs: cisplatin, BCNU, adriamycin and taxol were combined with MJ, and the cytotoxic activity of each combination was evaluated in six malignant cell lines including breast, lung, prostate and pancreas carcinomas as well as leukemia cells. MJ synergized with all four drugs, although the efficiency of the different combinations varied in the different cell types (*33*). Additional evidence for the benefit of combining MJ with cisplatin was demonstrated in a study by Yeruva at al (*34*). In that study, combination of MJ, cisplatin and perillyl alcohol, a naturally occurring monoterpene that inhibits growth of cancer cells and induces cell death, resulted in synergistic effects on growth inhibition of two different human breast carcinoma cell lines (*34*). In accordance with the results obtained in vitro, combined treatment with MJ and adriamycin in vivo significantly prolonged the survival of leukemia-bearing mice, as compared to treatment with each agent separately (*33*). In addition to its synergistic effect when combined with conventional chemotherapeutics, MJ also synergized with novel agents that are not yet used in routine treatment regimens. MJ synergized with the glycolysis inhibitor 2-deoxy-D-glucose (2-DG) (*25*, *33*) as well as with inhibitors of the phosphatidylinositol 3-kinase/AKT pro-survival pathway (*14*). The mechanism underlying the combined effect of MJ and 2-DG will be further discussed in the next section of this chapter.

It is now evident that the combination of MJ with different cytotoxic agents, either conventional chemotherapeutics, regulators of glycolysis or inhibitors of the PI3K/Akt pathway, can produce a synergistic cytotoxic effect, both in vitro and in vivo. The efficiency of these combinations may provide a basis for future clinical treatments which involve combination of MJ and different anti-cancerous agents. These combinations might help to overcome drug resistance and allow reducing the administered dose of each drug thus decreasing unwanted side effects.

The ability of jasmonates to selectively kill cancer cells, overcome multi-drug resistance and exhibit anti-cancer activities in vivo, provide a strong basis for future evaluation of their clinical potential as novel anti-cancer drugs. Recently, a first-in man trial was performed in order to evaluate the effect of MJ on various pre-malignant and malignant skin lesions (*21*). Eight patients with various skin lesions were chosen on a compassionate basis. Treated cases included: lichen planus orale, sebaceous perineal cancer, squamous cell cancer from lower leg ulcers, lentigo maligna of the face with mixed basal spinal cells carcinoma, relapsed precancerous lesion of perineal skin, meningioma with temporomandibular joint and submucosal parapharigeal diffusion, squamous cell cancer of arm and lower leg, and leukoplakia. None of the skin lesions could be surgically removed due to previous failure of a single or multiple surgeries, the extent of cancer or high risk of surgery. Additionally, all eight lesions proved resistant to different drug schedules, including chemotherapy or local ointments. MJ was administrated in oil suspension at 1 mg/ml. The compound was administered twice daily on the diseased skin or mucosa for 4 weeks with an oblcusive poliurethan medication in order to enhance the compound absorption. Three out of the eight patients exhibited positive responses to MJ treatment, i.e., the patient with lichen planus orale and the patient with leukoplakia, had complete recovery (for 18 months following the first treatment, at the time of paper submission), while MJ treatment of the patient with lentigo maligna of the face resulted in dry tumor surface with reduction of the metaplastic area during the treatment, but the cancer reappeared three months later. It is important to note that MJ was very well tolerated and no meaningful local or systemic side effects have been detected (*21*). This study provides the first evidance for the acitivity of MJ against human pre-cancerous and cancerous skin lesions and paves the way for additional, larger scaled clinical trials.

The data presented herein demonstrates that jasmonates exhibit anti-cancer activity both in vitro and in vivo and were lately shown to have an effect on human pre-cancerous and cancerous skin lesions. Jasmonates induce death and inhibit the proliferation of many cancer cell types including drug-resistant cells. Remarkably, jasmonates can also prevent the spread of metastasis by inhibiting cancer cell migration. In addition to their direct effect on cancer cells, jasmonates were also shown to perturb the angiogenic process, thus impairing the tumors' vasculature. Importantly, jasmonates are highly selective towards cancer cells and have little or no effect on normal cells creating a wide therapeutic window. The high selectivity of jasmonates towards cancer cells as well as their ability to overcome multi-drug resistance can both be explained by their unique mechanism of action.

Jasmonate Mechanism of Action

Before discussing the jasmonate mechanism of action we will discuss the signaling pathways and cellular events that mediate cell death. The two prominent mechanisms of cell death are necrosis and apoptosis. Necrotic cell death is characterized by cytoplasmic and organelle swelling, followed by the loss of cell membrane integrity and release of the cellular contents into the surrounding extracellular space. Necrosis is often a consequence of a pathophysiological condition, such as infection, inflammation or ischemia. Although, for many years, necrosis has been considered an accidental and uncontrolled form of cell death, accumulating evidence supports the notion that necrosis can also occur in a regulated and controlled manner (*35*, *36*). Apoptosis is a form of programmed cell death, a physiological 'cell-suicide' program, in which biochemical and morphological events are usually organized in a cascade of specific and controlled steps. Apoptosis is essential for embryonic development, immune-system function and the maintenance of tissue homeostasis in multicellular organisms (*35*). The beginning of apoptosis is marked by chromatin condensation that occurs in parallel to the shrinking of the cell. Following events are fragmentation of the nucleus, transformation of the cell surface, and finally complete splitting of the cell contents into apoptotic bodies attached to the membrane (*37–39*). These apoptotic bodies are then engulfed and cleared by phagocytes (*40*). The terminal events of apoptosis involve the activation of a series of cysteine aspartyl-specific proteases termed caspases (*39*). Activated effector caspases cleave a specific set of cellular substrates, resulting in the biochemical and morphological changes that are associated with the apoptotic phenotype (*41*). Caspase-3 is an effector

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caspase that is essential for a number of morphological and biochemical events associated with apoptosis (*42*).

Several signaling pathways can mediate the apoptotic process. Many of these signaling pathways converge on mitochondria and cause the permeabilization of the mitochondrial outer membrane, leading to the release of pro-apoptotic proteins such as cytochrome c from the mitochondria into the cytosol. These events further trigger the apoptosis process by activating caspase-9 and subsequently caspase-3 (*43*). Mitochondrial membrane permeabilization also impairs the bioenergetic function of mitochondria, promoting cell death due to ATP depletion (*44*).

Jasmonates were shown to induce both apoptotic and non-apoptotic cell death in cancer cells. Several mechanisms of action have been proposed to explain the anti-cancerous activity of jasmonates. These mechanisms are not mutually exclusive and may, therefore, either occur concomitantly or differ according to cell type, time frames or concentration range.

The Mitochondria-Toxic Mechanism

As mentioned above, the mitochondria play a pivotal role in life and death decisions in cells. In recent years, the mitochondria have become a target for anti-cancer therapies (*45*). Many chemotherapeutic drugs are designed to induce cell death by triggering the apoptotic machinery. Unfortunately, many proteins that participate in the apoptotic cascade are either mutated or deregulated in cancer cells, thus rendering many cytotoxic drugs ineffective (*46*). These mutations often occur upstream of the mitochondria (*44*). Since many signaling pathways that induce apoptosis converge on mitochondria, perturbing mitochondria directly, may bypass the blocks in the apoptotic machinery and enable the killing of otherwise resistant cells. Thus, the direct effect of jasmonates on mitochondria was evaluated. First, the effect of jasmonates on mitochondria from intact human leukemia and hepatoma cells was evaluated. Jasmonates were shown to perturb mitochondria in these cells causing cytochrome c release from the mitochondria into the cytosol and mitochondrial membrane depolarization (*23*). Then, the direct effect of MJ on mitochondria isolated from those cells was evaluated, measuring cytochrome c release from mitochondria and mitochondria swelling, reflecting mitochondrial membrane permeabilization. MJ directly perturbed the mitochondria isolated from both cells lines. The clinical relevance of the above mentioned findings was supported by similar experiments performed on ex vivo leukemic cells isolated from the blood of CLL patients. Jasmonates induced membrane depolarization in the leukemic cells, and induced swelling and release of cytochrome c in mitochondria isolated from these cells. The effect of MJ was selective to cancer cell mitochondria while mitochondria isolated from non-transformed 3T3 fibroblasts or normal lymphocytes were not affected (*23*). Mitochondrial membrane permeabilization can be induced by mitochondrial membrane permeability transition (MPT). MPT involves a prolonged opening of a channel named permeability transition pore complex (PTPC), the central components of which include the adenine nucleotide translocator, cyclophilin D, and the voltage-dependent anion channel (VDAC) (*47*, *48*). The effect of MJ on cancer cell mitochondria was shown to involve the opening of PTPC, as compounds that induce PTPC closure protected the mitochondria from MJ-induced damage (*23*). An additional evidence for the involvement of the mitochondria in the cytotoxic effect of MJ was found in a recent study that demonstrated that MJ induced mitochondrial membrane depolarization in HL-60 and KG-1 acute myelogenouse leukemia cell lines. Treatment of these two cell lines with MJ, led to an increase in mitochondrial superoxide (MSO) levels, possibly indicative of mitochondrial damage (*16*).

These results demonstrate that MJ can perturb mitochondria directly in a PTPC-dependant manner. The next step in revealing the mechanism by which MJ affects mitochondria was determining the molecular target underlying its mitochondria-toxic activity.

Hexokinase (HK) is the enzyme that catalyzes the first step of glycolysis, phosphorylating glucose to yield glucose-6-phosphate. In healthy cells, HK interacts with the PTPC through binding to VDAC, thereby inhibiting mitochondrial membrane permeabilization (*46*). Dissociation of hexokinase from VDAC may lead to mitochondrial perturbation and promotes cell death (*49–51*). In cancer cells, mitochondria-bound hexokinase and VDAC are overexpressed (*52*). Therefore, agents that interfere with HK-VDAC interaction may represent an efficient approach for killing cancer cells selectively with minimum effect on normal cells. A recent study identified HK as the molecular target of MJ (*53*). MJ was shown to bind directly to HK and detach it from VDAC and the mitochondria. The detachment of HK from mitochondria led to opening of the PTPC followed by cytochrome c release and swelling of the mitochondria (*53*). These results reveal the mechanism by which MJ induces its mitochondria-toxic effect which ultimately leads to cell death.

Mitochondrial oxidative phosphorylation is a major source of cellular ATP. In normal cells, mitochondrial oxidative phosphorylation is responsible for producing most of the ATP required for fundamental cellular processes. However, a different picture can be seen in cancer cells. Cancer cells preferentially utilize glycolysis for production of ATP, even when oxygen is prevalent, a phenomenon known as the Warburg effect (*54*). Despite the great deal of significance that has been attributed to cancer cell glycolysis, mitochondrial oxidative phosphorylation is still crucial for sufficient supply of ATP in cancer cells (*54*, *55*). The aberrant function of mitochondria in cancer cells may render them the ‘achilles’ heel’ of these cells (*44*). In accordance with the effect of MJ on cancer cell mitochondria, treatment with MJ induced a rapid decrease in cellular ATP levels in various caner cell lines. MJ induced a drop in cellular ATP levels within 15 minutes, hours before any sign of cytotoxic effect could be recorded (*10*, *12*, *13*, *33*). Again, the effect of MJ was selective towards cancer cells, while normal lymphocytes did not exhibit ATP depletion following MJ treatment (*10*). Given the fact that jasmonates perturb cancer cell mitochondria and induce a rapid decrease in cellular ATP levels, the combined effect of MJ and the glycolysis inhibitor 2-DG on the viability and ATP levels of several cancer cell lines was evaluated. MJ significantly enhanced 2-DG-mediated ATP depletion and/or cytotoxicity, resulting in a synergistic effect, in several cancer cell lines including B-lymphoma, colon carcinoma, lung carcinoma and breast adenocarcinoma cells (*25*, *33*). Moreover, high levels of glucose protected fibrosarcoma and lymphoma cells from MJ-induced ATP depletion or cytotoxicity (*14*, *25*). The additive effect of MJ and 2-DG implies that the effect of the two compounds on ATP levels are mediated via inhibition of different mechanisms of ATP synthesis. In line with this conclusion, neither an inhibitor (oligomycin) nor a substrate (pyruvate) of oxidative phosphorylation could influence the effect of MJ on ATP levels (*25*). This fact supports the conclusion that MJ compromises the oxidative phosphorylation pathway which could not be further compromised by oligomycin. 2-DG is a metabolic inhibitor of HK. The fact that HK is a common target for both MJ and 2-DG may underlie their synergistic effects on ATP depletion and cell viability.

The direct effect of MJ on mitochondria may explain its cytotoxic activity against drug resistant cells. Since the apoptotic signaling cascade is often aberrant in cancer cells, the fact that MJ perturbs mitochondria directly may enable it to bypass the apoptotic block and induce death in otherwise resistant cells. As mentioned earlier in this chapter, MJ was able to overcome drug resistance induced by p53-mutation. Interestingly, just like the cytotoxic drugs bleomycin and NCS, MJ induced apoptosis in the wild-type p53 expressing cells. However while the mutant-p53 expressing cells harbor an aberrant apoptotic cascade that rendered them resistant to bleomycin and NCS, MJ could bypass this apoptotic block by inducing a non-apoptotic cell death in these cells (*25*).

The ability of MJ to perturb the mitochondria of cancer cells while sparing mitochondria from non-transformed cells is in line with the fact that mitochondria from normal and cancer cells may differ in composition and function. Cancer cells are characterized by higher mitochondrial membrane potential, modulation of the expression of PTPC components, and reduced expression of the β-catalytic subunit of the mitochondrial H+-ATP synthase. Several mutations in mitochondria and nuclear DNA affect components of the mitochondrial respiratory chain resulting in insufficient ATP production, and reactive oxygen species (ROS) overproduction, ensuing in oxidative damage. Moreover, as previously mentioned, mitochondria-bound hexokinase and VDAC are overexpressed in cancer cells (*46–48*, *52*, *56*). Thus, these differences between normal and cancer cell mitochondria may lay the basis for the selective effect of MJ on cancer cell mitochondria and consequently may explain the selective effect of jasmonates on cancer cells.

The Reactive Oxygen Species (ROS)-Mediated Mechanism

Many studies have demonstrated the cytotoxic effects of various ROS, such as hydrogen peroxide, superoxide ion and singlet oxygen, towards cancer cells. The involvement of ROS in MJ-induced cytotoxicity was, therefore, also evaluated. Studies performed on A549 lung carcinoma cells revealed that MJ-induced apoptosis in these cells involves the production of hydrogen peroxide followed by increased expression of pro-apoptotic proteins of the Bcl-2 family, Bax and Bcl-Xs (*11*). The anti-oxidant catalase, which decomposes hydrogen peroxide, protected the cells against the MJ-induced increase in the level of Bcl-2 family members and cells death. Thus, induction of apoptosis in A549 cells appears to be mediated by a cascade involving hydrogen peroxide generation and an increase in the expression of pro-apoptotic proteins of the Bcl-2 family (*11*).

In a different set of experiments, MJ was shown to induce heat shock protein 72 (HSP72) in C6 glioma cells via heat shock factor I. MJ-induced expression of HSP72 was prevented by specific inhibition of hydrogen peroxide and hydroxyl radicals (*15*). Finally, treatment of HL-60 and KG-1 acute myelogenous leukemia cells with JA or MJ led to an increase in ROS levels (*16*). These data indicate that ROS facilitate the cytotoxic activity of MJ through several mechanisms.

Interestingly, similar results were obtained with plant cells. MJ induced mitochondrial ROS production, followed by loss of mitochondrial transmembrane potential and mitochondrial swelling in cells of the Arabidopsis thaliana plant. Cell death followed mitochondrial perturbation. The anti-oxidants ascorbic acid or catalase prevented MJ-induced ROS production and subsequent cell death. Moreover, the PTPC inhibitor cyclosporin A also gave significant protection against MPT loss, mitochondrial swelling and subsequent cell death (*5*). These results further demonstrate that jasmonates may mediate similar functions in plants and animal cells.

The Re-Differentiation Mechanism

One of the main unique characteristics of cancer cells is the low level of differentiation they exhibit, which allows them to obtain a malignant phenotype. The concept of inducing re-differentiation as a strategy to "normalize" undifferentiated cancer cells has attracted cancer scientists for many years. MJ induced the differentiation of several human leukemic cell lines including HL-60 human myeloid leukemia cells, thus inhibiting their proliferation (*17*). It is important to note that the effect of MJ on the differentiation of the leukemic cells was obtained under concentrations and time frames that did not induce cell death. Several markers of differentiation were induced by MJ in HL-60 cells. Those included: NBT-reduction (a typical marker of myelomonocytic differentiation), morphologic differentiation into granulocytes with some properties of monocytes such as monocytic granules and expression of both monocyte-specific surface antigen CD14 and granulocyte-specific antigen CD15. Finally, MJ induced α-naphthyl acetate esterase activity, a marker of monocytic differentiation. Known inducers of granulocytic and monocytic differentiation, all-trans retinoic acid and 1α,25-dihydroxyvitamin D3, respectively, synergized with MJ in the induction of HL-60 differentiation. The differentiation induced by MJ in HL-60 cells was mediated by activation of mitogen activated protein kinase (MAPK) and could be inhibited by the MAPK inhibitor PD98059 (*17*). In addition, MJ induced the expression of the calcium-binding protein, and inducer of cell differentiation, S100P. MJ-induced upregulation of S100P was in correlation with its growth–inhibitory activity. A microarray expression assay revealed that the expression profile induced by MJ resembles that induced by IPA, a known inducer of differentiation, suggesting that these inducers share common signal transduction systems for inducing the differentiation of leukemia cells (*18*). Another naturally occurring jasmonate, methyl 4,5-didehydrojasmonate (Me-DDHJ) was about 30 times more potent than MJ in inducing the differentiation of HL-60 cells. Additionally, Me-DDHJ significantly stimulated both functional and morphological differentiation of leukemia cells that had been freshly isolated from patients with acute myeloid leukemia (*18*). Notably, the naturally occurring (+)-Me-DDHJ proved to be more potent than the unnatural (-)-Me-DDHJ isomer in inducing morphological differentiation and inhibiting the growth of HL-60 cells and leukemic cells drawn from acute myeloid leukemia patients. The differential activity of the two isomers was confirmed by their effects on the expression of differentiation-associated antigens and induction of NBT-reduction, with the natural (+)-Me-DDHJ being more potent than the unnatural isomer (*18*). The effect of Me-DDHJ on the differentiation of HL-60 cells was mediated by MAPK indicating that its mechanism of action might be similar to that of MJ (*18*). However, the effect of Me-DDHJ on the expression of S100P remains to be evaluated.

AKR1C Inhibition Mechanism

Members of the aldo-keto reductase (AKR) superfamily, particularly the AKR1C subfamily, are emerging as important mediators of cancer pathology. Specifically, AKR1C3 polymorphisms have been shown to modulate risk of various cancers, including lung carcinoma, childhood leukemias, diffuse large B-cell lymphoma, and carcinomas of the prostate and bladder (*58–61*). AKR1C3 was shown to regulate myeloid cell differentiation. Overexpression of AKR1C3 suppresses the differentiation of HL-60 human myelogenous leukemia cells, while inhibition of AKR1C3 triggers the differentiation of these cells into neutrophils or monocyes (*62–64*). Thus, compounds that inhibit AKR1C enzymes and AKR1C3 in particular, may provide novel agents for either the chemoprevention or treatment of diverse malignancies. As AKR1C3 is known to play a part in the metabolic conversion of prostaglandin D2 into 9α,11β-prostaglandin F2α and given the structural similarity between prostaglandins and jasmonates, Davies et al. examined the plausibility of AKR1C3 as a target for MJ (*16*). Interestingly, this group found that JA and MJ are capable of inhibiting the activity of all four human AKR1C isoforms both in vitro and in an acute myelogenous leukemia cellular model (*16*). Given AKR1C3's association with cancer biology and cell differentiation, this inhibition could potentially contribute to the anti-cancer effects of jasmonates.

Apoptosis Modulation Mechanism

Several groups have reported that jasmonates can either activate pro-apoptotic proteins or inhibit anti-apoptotic proteins, thus leading to apoptotic cell death. Three naturally occurring jasmonates, MJ, JA and cis-jasmone (Figure 1) were shown to induce apoptosis in SH-SY5Y neuroblastoma cells (*20*). MJ was also shown to induced apoptosis in SK-N-SH and BE(2)-C neuroblastoma cells (*19*). Jasmonate treated cells exhibited nuclei condensation, eccentric nuclei, and expression of phosphatidylserine on the outer leaflet of the cell membrane, all of which are known markers of apoptosis. The IAP (inhibitors of apoptosis) family plays a role in oncogenesis by suppressing apoptosis. Treatment of SH-SY5Y neuroblastoma cells with MJ downregulated the expression of XIAP (X-linked inhibitor of apoptosis protein) and survivin, two important members of the IAP family. As previous studies have indicated that XIAP protects neuroblastoma cells against death induced by cytotoxic agents or growth factors withdrawal (*65*), the fact that MJ downregulates XIAP might not only trigger the apoptotic machinery but may also sensitize cancer cells to the effect of cytotoxic drugs. In addition to induction of apoptosis, MJ also inhibited the proliferation of SH-SY5Y neuroblastoma cells. MJ-induced cell cycle arrest in these cells was associated with down-regulation of c-myc and proliferating cell nuclear antigen (PCNA). Both are known inducers of cellular proliferation (*20*).

A study performed on human breast cancer cell lines, MDA-MB-435 and MCF-7, revealed that MJ induced apoptosis in the two cell lines (*9*). MJ-induced apoptosis, in these cells, was associated with a decrease in membrane fluidity and expression of TNFR1 followed by caspases-8, MAPK and caspase-3 activation, indicative of extrinsic apoptotic signaling (i.e., apoptosis triggered by engagement of cell surface receptors). Similar results were obtained in a study performed on two prostate cancer cell lines, PC-3 and DU-145 (*66*). In line with the results obtained with breast cancer cells, MJ-induced apoptosis in the two prostate cell lines was associated with TNFR1 expression and caspase-3 activation.

As mentioned above, in the human lung carcinoma cell line A549 MJ induced the production of hydrogen peroxide which led to increased expression of pro-apoptotic proteins of the Bcl-2 family, Bax and Bcl-Xs (*11*). The results described above demonstrate that MJ is able to regulate different proteins that mediate the apoptotic cascade, thus triggering apoptotic cell death.

Novel Jasmonate Derivatives – Biological Activity and Mechanism of Action

The concentrations of JA and MJ required to effect cell viability, proliferation, differentiation and migration are in the lower milimolar range 0.25-5 mM (*8*, *12*, *17*, *53*). These concentrations are considered relatively high compared to most common chemotherapeutics. Two approaches have been taken in order to reduce the effective concentration of jasmonates. The first was the administration of jasmonates in combination with different chemotherapeutics triggering a synergistic affect. This approach was extensively discussed earlier in this chapter. The second approach was to synthesize new chemical derivatives with improved anti-cancer activity. A number of research groups have taken the natural jasmonate compounds as a starting point to prepare and biologically evaluate a wide variety of jasmonate derivatives. Several of these derivatives showed an enhanced ability to induce apoptosis, cell cycle arrest or differentiation in different cancer cell lines. As halogenated derivatives of MJ were shown to exhibit superior activity in plant systems (*65*), we have synthesized five halogenated jasmonate derivatives containing Br, I or F atoms and evaluated their cytotoxic activity in Molt-4 leukemic cells. We found that all of the novel compounds exhibited superior cytotoxic activity in Molt-4 cells, compared to MJ (*12*). However, Compound I, a 5,7,9,10-tetrabromo derivative of MJ (Figure 1), was by far the most active derivative, with an IC50 which is approximately 55 times lower than that of MJ. Compound I is a mixture of 2 isomers Ia and Ib at a 1:1 ratio (Figure 1). It is interesting to note that both isomers were equally cytotoxic towards Molt-4 cells. The cytotoxicity of compound I was further evaluated in B16-F10 melanoma, MCF-7 breast carcinoma, MiaPaCa-2 pancreas carcinoma and D122 lung carcinoma cell lines. Compound I was significantly more active than MJ, in each of the cancer cell lines tested (*12*).

Importantly, just like MJ, compound I exhibited selective cytotoxicity towards cancer cells while sparing normal blood lymphocytes (*12*). As MJ exhibited anti-migratory and anti-metastatic effects in B16-F10 melanoma cells, the ability of compound I to suppress the metastatic process in these cells was evaluated. Compound I did not inhibit the migration of B16-F10 cells. However, it inhibited the adhesion of B16-F10 cells to the substratum. Adhesion of cancer cells to their substratum is another important component of the metastatic process (*24*). Accordingly, Compound I inhibited the development of melanoma metastases in the lungs of mice, thus exhibiting an anti-metastatic activity in vivo. Compound I inhibited metastasis in vivo when administrated at 20 mg/Kg, while MJ exhibited the same effect only when administrated at 70 mg/Kg (40 mg/Kg proving ineffective) (*12*). Thus, compound I proved to be more potent than MJ in suppressing melanoma metastasis in vivo. The mechanism of action of MJ and compound I, underlying their anti-metastatic activities, remains to be elucidated. However, the fact that MJ inhibited melanoma cell migration while compound I inhibited melanoma cell adhesion but not migration, suggests that the two compounds do not share a common anti-metastatic mechanism of action.

Several additional jasmonate derivatives, including some halogenated derivatives, proved more potent then MJ in inducing the differentiation of HL-60 myeloid leukemia cells (*17*). As mentioned earlier, the introduction of a double bond at the 4,5-position of MJ, generating the natural derivative Me-DDHJ, greatly enhanced the differentiation-inducing activity of MJ (*17*). This chemical modification, altering the cyclopentanone moiety of MJ to yield a cyclopentenone moiety, was the basis for the synthesis of another potent jasmonate derivative, methyl 5-chloro-4,5-didehydrojasmonate (MeCl-DDHJ) (J7) (Figure 1). It is interesting to note, that MeCl-DDHJ (J7) is also a halogenated derivative of MJ, containing a Cl atom. This fact further validates the promising anti-cancer potential of halogenated jasmonate derivatives. MeCl-DDHJ at a concentration of 0.05 mM induced about 50% cytotoxicty in hepatoma cells after 24 h, while the concentrations of MJ required in order to induce the same extent of cyotototoxicity in the different cancer cell lines tested were around 1-3 mM. MeCl-DDHJ induced apoptosis in hepatoma cells by modulating several apoptosis-related proteins. It induced the expression of the pro-apoptotic protein death receptor 5, and downregulated the expression of the anti-apoptotic proteins XIAP, c-IAP-1 and Bcl-2. These events were associated with activation of caspase-8, caspase-9 and caspase-3 and resulted in apoptotic cell death (*67*). Another study demonstrated the apoptotic activity of MeCl-DDHJ on human cervical carcinoma cells. Treatment of HeLa cervical carcinoma cells with MeCl-DDHJ for 48 hours yielded a dose dependent decrease in viability with an IC50 of about 15 μM (*68*). The IC50 of MJ as determined in HeLa cell treated with MJ for 24 hours was about 3 mM (*13*). Although a longer incubation with MJ may have reduced its IC50 value, it is still obvious that MeCl-DDHJ exhibits a significantly higher cytotoxicity in HeLa cells, compared to MJ. The apoptotic activity of MeCl-DDHJ in Hela cervical carcinoma cells was associated with downregulation of the anti-apoptotic protein Bcl-2 and activation of caspase-3 and caspase-9 (*68*). MeCl-DDHJ as well as Me-DDHJ contain a cyclopentenone moiety. The introduction of cyclopentenone into anti-cancer molecules was shown to increase their potency. This has been shown for several molecules including chalcones (*7*, *69*). Furthermore, various compounds within the cyclopentenone prostaglandin family possess potent anti-inflammatory and anti-neoplastic activities. Most actions of the cyclopentenone prostaglandins do not appear to be mediated by binding to prostanoid receptors. Rather, some of the activities of these compounds appear to be mediated by the reactive α,β-unsaturated carbonyl group located in the cyclopentenone ring (*70*). Actually, some of the biological activities of cyclopentenone prostaglandins can be mimicked by the simple model compound 2-cyclopentenone (Figure 1) itself, although at 30- to 100 fold higher concentrations (*7*, *71*). From a biochemical point of view, the α,β-unsaturated carbonyl group of cyclopentenone is an electrophilic center susceptible to undergoing addition reactions with nucleophiles such as free sulfydryl groups of reduced glutathione or cysteine residues in proteins. Alkylation of crucial cysteine residues can result in a loss of function of the target proteins (*70*). As the α,β-unsaturated carbonyl group is a ‘soft’ electrophile, alkylation of weaker nucleophilic sites located in other macromolecules such as DNA, is less likely (*72*). Overall, it is evident that both the introduction of a double bond into the cyclopenatanone ring, yielding a cyclopentenone moiety, as well as the addition of halogen atoms, increases the activity of MJ.

Additional Activities of Jasmonates

While the vast majority of studies on jasmonates as potential drugs have been performed in the cancer arena, these compounds have also been evaluated as anti-parasitic and anti-inflammatory agents.

Two major human blood parasites, Plasmodium falciparum, a unicellular parasite causing malaria, and Schistosoma mansoni, a multicellular helminth parasite, were studied, and the effects of jasmonates on these parasites in vitro were determined. It was found that jasmonates are cytotoxic toward both parasites, with P. falciparum being the more susceptible. Of note, jasmonates did not cause any damage to control human erythrocytes (the host cells of the malaria parasites) at the maximum concentration used in the experiments (*73*). This was the first study demonstrating the anti-parasitic potential of plant-derived jasmonates. A third parasite whose susceptibility to jasmonates was studied infects the human urogenital tract, i.e., Trichomonas vaginalis. MJ induced death of T. vaginalis and fragmentation and condensation of its DNA, resembling phenomena associated with apoptotic death. However, DNA laddering, a sub-G1 cell cycle stage peak, and caspase-3 activation were not observed. Thus, MJ-induced T. vaginalis cell death appears to be non-apoptotic. Furthermore, MJ induced cell cycle block at the G2/M phase in T. vaginalis, similar to the effect of metronidazole, the drug of choice for the treatment of trichomoniasis. Finally, MJ was found to be cytotoxic towards a metronidazole-resistant strain of T. vaginalis, suggesting that it may be effective for the treatment of nitroimidazole-refractory trichomoniasis (*74*).

As mentioned above, MJ has direct mitochondriotoxic effects, strongly suggesting that mitochondria are target organelles of jasmonates. In this context, another conclusion of the T. vaginalis study was that jasmonates are able to damage cells lacking mitochondria, i.e., T. vaginalis. Examination of the influence of MJ on the bioenergetic pathways of T. vaginalis, indicated that depletion of ATP did not precede death of the parasites, but rather reflected it, in contrast to the case with cancer cells. Nevertheless, 2-DG, a glycolysis blocker, was synergistic with MJ in causing death of T. vaginalis cells, suggesting that MJ does perturb the bioenergetic homeostasis of the parasites, although mitochondria are not necessarily its target organelles (*74*). A recent study (*75*) demonstrated effects of MJ on the hydrogenosomes (ATP-producing, mitochondrion-like organelles present in T. vaginalis). Morphologically, after MJ treatment, the hydrogenosomes were no longer aligned with the axostyle, but spread out into the cytoplasm, and some of the hydrogenosomes showed low electron density. Functionally, MJ treatment induced a reduction in hydrogenosomal membrane potential, indicating that MJ might induce parasite death by damaging the hydrogenosomes. Thus, it appears that the anti-Trichomonas activity of MJ is at least associated with damage to an ATP-producing organelle, similar to its anti-cancer mode of action.

As mentioned above, introduction of a double bond into the cyclopentanone ring of MJ yielding the cyclopentenone Me-DDHJ significantly increases the cytotoxic activity of MJ. Actually, it was recently found that unsaturated ketones (enones) functionality is essential for the anti-inflammatory activity of certain compounds (*76*). In a quest for new leads with the enone functionality, and higher potency than natural anti-inflammatory prostaglandins (PGA1, PGA2, and 15-deoxy-D2,14-PGJ2), MJ which shares partial structure with anti-inflammatory prostaglandins, was selected as a starting material for the synthesis of cyclopentenone prostaglandin-like compounds. Eighteen MJ analogues were synthesized and evaluated for their inhibitory effects on the production of pro-inflammatory mediators (NO, IL-6, and TNFα) in lipopolysaccharide (LPS)-activated RAW264.7 mouse macrophage cells. The introduction of an enone functionality to the structure of MJ rendered the product, Me-DDHJ, a significant anti-inflammatory activity. Several halogenated derivatives further derived from Me-DDHJ, (MeCl-DDHJ, Methyl 5-bromo-4,5-didehydrojasmonate, Methyl 5-chloro-4,5-didehydro-dihydrojasmonate and Methyl 5-bromo-4,5-didehydro-dihydrojasmonate – Figure 1) exhibited even more enhanced activity, and these compounds were much more potent than the abovementioned natural anti-inflammatory prostaglandins. Among them, Methyl 5-bromo-4,5-didehydrojasmonate and Methyl 5-bromo-4,5-didehydro-dihydrojasmonate showed the highest potency, while MeCl-DDHJ and Methyl 5-chloro-4,5-didehydro-dihydrojasmonate would be more desirable with respect to safety. These results suggest that alpha-haloenone jasmonates may serve as potential anti-inflammatory leads (*77*). A recently published article revealed that the anti-inflamattory activity of Me-DDHJ was mediated through inhibition of the NF-kB pathway as well as downregulation of the microRNA molecule miR-155 (*78*). It is interesting to note that chronic inflammation is generally considered to be a pro-carcinogenic state. There are numerous explanations for this assertion, e.g., the generation by inflammatory cells of oxygen intermediates, known to induce genetic mutations. Thus, compounds that share the ability to both suppress tumor cell growth, and inflammatory cell function, are in effect multi-faceted preventive anti-cancer agents. Indeed, MeCl-DDHJ has both potent anti-inflammatory activities (*77*), as well as anti-cancer activities (*68*).

Overall, the data presented herein demonstrates that natural and synthetic jasmonates are endowed with anti-cancer activity. These results lay the basis for further research that may lead to the development of jasmonates as anti-cancer drugs.

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Chapter 18

Chemical Composition and Biological Effects of Maple Syrup

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Pure maple syrup is unique in that it is the largest commercially produced and consumed plant natural product that is obtained entirely from the sap of trees. It is a natural sweetener produced by concentrating the colorless watery sap collected from certain maple (genus, *Acer*) species. The natural maple tree sap contains minerals, oligosaccharides, peptides, amino and organic acids, phytohormones, and phenolics, apart from sucrose which is its predominant sugar. During the intensive heating process required to transform sap to syrup, a complex cocktail of both native (originally present in the xylem sap), and derived (formed through chemical reactions during processing) phenolic compounds ultimately ends up in maple syrup. From a human health perspective, this is interesting considering that phenolics have attracted significant research attention for their potential role in the prevention and treatment of several chronic human diseases. Here we review current scientific knowledge of the chemical constituents, in particular phenolics, present in maple syrup. The biological activities of these compounds, in relation to the health benefits that may result from maple syrup consumption, are also briefly discussed.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Introduction

Plant foods and their derived products have attracted tremendous scientific attention for their biological effects and possible human health benefits. While plants contain several essential macro- and micronutrients, such as protein, carbohydrates, fat, fiber, minerals and vitamins, research has shown that their secondary metabolites i.e. phytochemicals, may impart health benefits beyond basic nutrition. Moreover, it is now well accepted that consumption of a phytochemical-rich diet contributes towards reducing the risk of several oxidative-stress and inflammatory mediated diseases such as certain cancers, heart, and other chronic human illnesses.

Phytochemical-rich foods include fruits, vegetables, whole grains, spices, beverages, and their derived products such as juices, tea, coffee and wine. Interestingly, among plant foods, maple syrup, which is the subject of this review, stands out in that it is the largest commercially available and consumed natural product which is obtained entirely from the sap of deciduous trees.

Maple syrup is a natural sweetner produced by concentrating the sap collected from certain maple species (genus, *Acer*) (*1*, *2*). The main maple species used for this purpose include the sugar maple (*A. saccharum*), red maple (*A. rubrum*), and black maple (*A. nigrum*) trees which are all native to North America (*1*, *2*). These maple species are best utilized for maple syrup production because their sap contains a higher sugar content compared to that of other maples (*1*). Maple sap is collected in the late winter to spring months when freeze/thaw cycles causes the sweet sap to rise and flow from taps made in the tree trunk (*1*, *2*).

Maple syrup is primarily produced for commercial purposes in the north-eastern regions of Canada and the United States. Remarkably, Canada (85%; mainly the province of Quebec) leads the world production of maple syrup. Thus, apart from its economic importance, maple syrup production is of great cultural significance to this region of the world. Maple syrup is obtained by the thermal evaporation of the colorless watery sap and about 40 L of sap is required to produce 1 L of MS (*1*). During the concentration process of transforming sap to syrup, the characteristic flavor, color, and odor of MS develops. Typically, the color of the syrup becomes darker as the season progresses, and based on Canadian standards, MS is graded as extra light (grade AA), light (grade A), medium/amber (grade B), and dark (grade C) (*2*).

Given the worldwide popularity and consumption of maple syrup, knowledge of its chemical constitutents is of great scientific interest. This is especially relevant from a human health perspective given the aforementioned attention that phytochemicals have attracted. Recent research published by our laboratory (*3*, *8*), and others (*4*–*7*), have revealed a wide diversity of phenolic compounds present in pure maple syrup. Here we review the chemical compounds, particularly phenolics, identified in maple syrup, to date, and discuss the biological effects and potential health benefits that may result from maple syrup consumption. It should be noted that certain constituents of maple syrup, specifically minerals, monosaccharides and oligosaccharides, organic and amino acids, peptides, and proteins are well established (*1*, *2*). Thus, this review primarily focuses on the phytochemical constituents present in maple syrup, specifically those which are phenolic in nature.

Chemical Constituents of Maple Syrup

The chemistry of maple syrup is complicated due to the presence of naturally occurring compounds, originally present in the xylem sap, as well as process-derived compounds which are formed during the intensive heating process required to transform sap into syrup. While increasing the complexity of the final product, these *in situ* reactions also give maple syrup its characteristic color, odor, and flavor (*1*). Ultimately, due to a combination of these factors, a complex cocktail of compounds ends up in maple syrup. It should be noted that the chemistry of maple syrup may be further complicated depending on the geographical location and the particular maple species (or combination thereof) used for sap collection, etc.

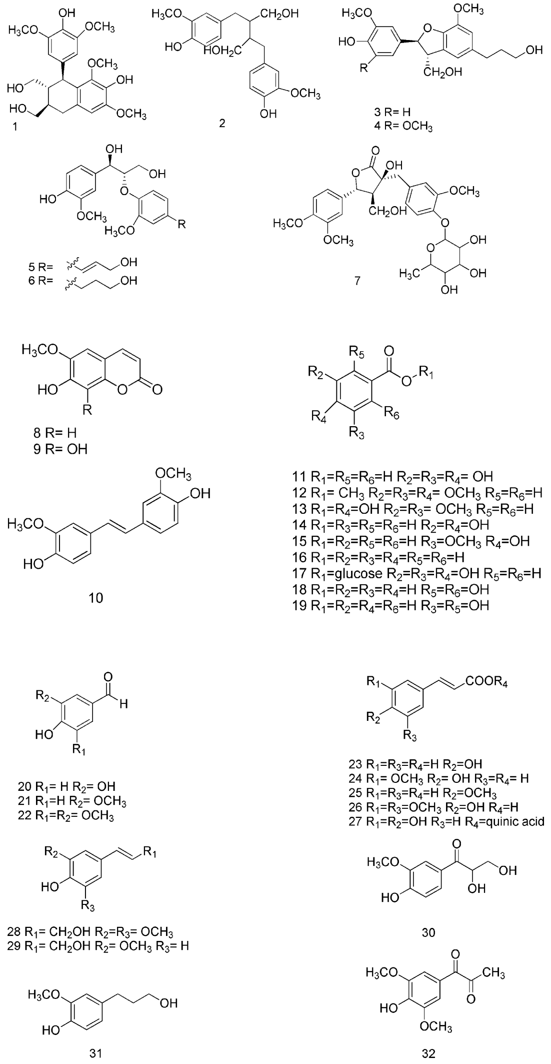
To date, the chemical constituents reported in maple syrup are shown in Table 1 and the corresponding structures of these compounds are shown in Figure 1. The majority of the compounds reported in maple syrup are phenolic in nature and include lignans (**1-7**), coumarins (**8-9**), stilbene (**10**), benzoic acid (**11-19**) and benzaldehyde (**20-22**) derivatives, phenylpropanoids (**23-32**), and flavonoids (**33-39**). Apart from these phenolic compounds, several nitrogen containing molecules, namely pyrazines (**47-55**), and an assortment of other compounds (**56-69**), have also been reported in maple syrup.

Maple syrup is produced under intensive heating conditions required to transform sap to syrup. Thus, it is not surprising that maple syrup contains naturally occurring phenolics (present in the xylem sap) as well as non-natural, artefacts or process-derived compounds (formed by chemical reactions during its production). In addition, Maillard reactions occur between amino acids and reducing sugars, and polycarbonyl compounds are formed (*1*).

Apart from the natural plant phenolics reported in maple syrup, we have recently reported the isolation of a non-natural, process-derived phenolic compound in maple syrup from Canada (*8*). The compound, 2,3,3-tri-(3-methoxy-4-hydroxyphenyl)-1-propanol (**70**) was assigned the common name of quebecol. Examination of the maple sap using LC-MS analyses failed to identify quebecol therein, confirming that it is formed during the syrup and/or extract preparation (*8*).

Phenolics are the predominant phytochemical constituents found in maple syrup. They are also among the most ubiquitous and abundant phytochemicals present in plant foods and thus, in human diet. These compounds have attracted significant research attention for their biological activities, in particular their role as antioxidants and activities beyond antioxidation (reviewed in (*9*, *10*)). The role of these compounds in imparting potential biological effects and health benefits to maple syrup is further discussed below.

Naturally occurring plant phenolics exhibit considerable structural diversity including varying types and levels of oxidation as well as varying substitution patterns of hydroxylation and glycosylation. The main classes of monomeric natural phenolics that are commonly found in plant foods include flavonoids, lignans, stilbenes, coumarins, and phenolic acids (*9*). However, it should be noted that process-derived compounds can also be formed from reactions occurring among these compounds (*8*). Interestingly, maple syrup contains a cocktail of all of the aforementioned sub-classes of naturally occurring phenolics several of which are thought to be formed as degradation products of lignin components in sap (*1*). Thus, further research to comprehensively isolate and elucidate the structures of all of the chemical constituents present in maple syrup is warranted.


1
2
3 R= H
4 R= OCH3
5 R=
6 R=
7
8 R= H
9 R= OH
10
11 $R_1=R_5=R_6=H$ $R_2=R_3=R_4=OH$
12 $R_1=CH_3$ $R_2=R_3=R_4=OCH_3$ $R_5=R_6=H$
13 $R_1=R_4=OH$ $R_2=R_3=OCH_3$ $R_5=R_6=H$
14 $R_1=R_3=R_5=R_6=H$ $R_2=R_4=OH$
15 $R_1=R_2=R_5=R_6=H$ $R_3=OCH_3$ $R_4=OH$
16 $R_1=R_2=R_3=R_4=R_5=R_6=H$
17 R_1=glucose $R_2=R_3=R_4=OH$ $R_5=R_6=H$
18 $R_1=R_2=R_3=R_4=H$ $R_5=R_6=OH$
19 $R_1=R_2=R_4=R_6=H$ $R_3=R_5=OH$
20 $R_1=H$ $R_2=OH$
21 $R_1=H$ $R_2=OCH_3$
22 $R_1=R_2=OCH_3$
23 $R_1=R_3=R_4=H$ $R_2=OH$
24 $R_1=OCH_3$ $R_2=OH$ $R_3=R_4=H$
25 $R_1=R_3=R_4=H$ $R_2=OCH_3$
26 $R_1=R_3=OCH_3$ $R_2=OH$ $R_4=H$
27 $R_1=R_2=OH$ $R_3=H$ R_4=quinic acid
28 $R_1=CH_2OH$ $R_2=R_3=OCH_3$
29 $R_1=CH_2OH$ $R_2=OCH_3$ $R_3=H$
30
31
32

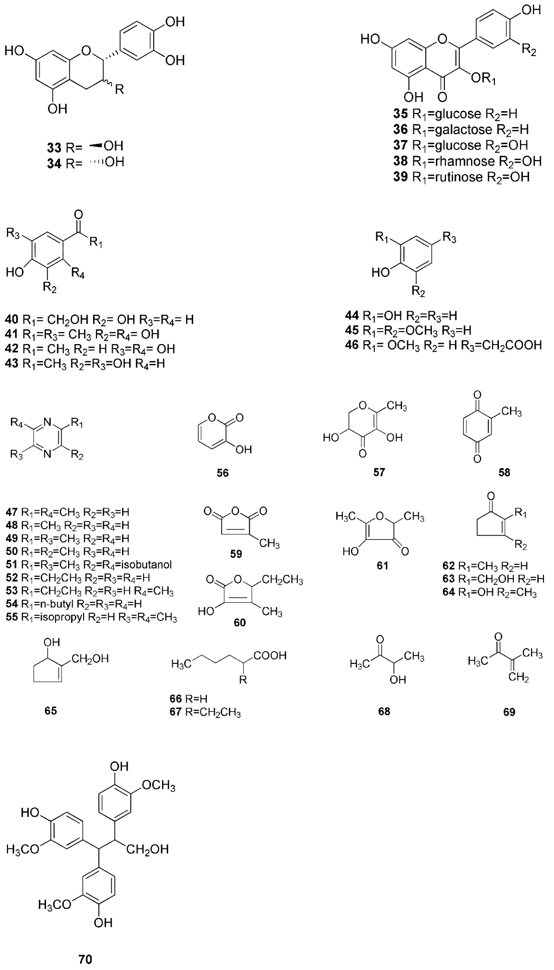

33 R= ◄OH
34 R= ''''OH
35 R_1=glucose R_2=H
36 R_1=galactose R_2=H
37 R_1=glucose R_2=OH
38 R_1=rhamnose R_2=OH
39 R_1=rutinose R_2=OH
40 R_1= CH_2OH R_2= OH R_3=R_4= H
41 R_1=R_3= CH_3 R_2=R_4= OH
42 R_1= CH_3 R_2= H R_3=R_4= OH
43 R_1=CH_3 R_2=R_3=OH R_4=H
44 R_1=OH R_2=R_3=H
45 R_1=R_2=OCH_3 R_3=H
46 R_1= OCH_3 R_2= H R_3=CH_2COOH
47 R_1=R_4=CH_3 R_2=R_3=H
48 R_1=CH_3 R_2=R_3=R_4=H
49 R_1=R_3=CH_3 R_2=R_4=H
50 R_1=R_2=CH_3 R_3=R_4=H
51 R_1=R_3=CH_3 R_2=R_4=isobutanol
52 R_1=CH_2CH_3 R_2=R_3=R_4=H
53 R_1=CH_2CH_3 R_2=R_3=H R_4=CH_3
54 R_1=n-butyl R_2=R_3=R_4=H
55 R_1=isopropyl R_2=H R_3=R_4=CH_3
56
57
58
59
60
61
62 R_1=CH_3 R_2=H
63 R_1=CH_2OH R_2=H
64 R_1=OH R_2=CH_3
65
66 R=H
67 R=CH_2CH_3
68
69
70

Figure 1. Structures of chemical constituents identified in maple syrup. Compounds 1- 46, and 70 are phenolics and compounds 47-69 are non-phenolic constituents (see Appendix C for larger version of figure).

Table 1. Chemical constituents identified in maple syrup

	Name	*Molecular Formula*	*Molecular weight*	*Reference*
	Lignans			
1	Lyoniresinol	$C_{22}H_{28}O_8$	420	(*3*)
2	Secoisolariciresinol	$C_{20}H_{26}O_6$	362	(*3*)
3	Dihydrodehydrodiconiferyl alcohol	$C_{20}H_{24}O_6$	360	(*1*)
4	5-methoxy-trans-dihydrodehydrodiconiferyl alcohol	$C_{21}H_{26}O_7$	390	(*3*)
5	Guaiacylglycerol β-coniferyl ether	$C_{20}H_{24}O_6$	360	(*3*)
6	Guaiacylglycerol-β-O-4′-dihydroconiferyl alcohol	$C_{20}H_{26}O_7$	378	(*3*)
7	3-[(4-[(6-deoxy-α-L-mannopyranosyl)oxy]-3-methoxyphenyl)-5-(3,4-dimethoxyphenyl)dihydro-3-hydroxy-4-(hydroxymethyl)-2(3H)-furanone	$C_{27}H_{34}O_{12}$	550	(*3*)
	Coumarins			
8	Fraxetin	$C_{10}H_8O_5$	208	(*3*)
9	Scopoletin	$C_{10}H_8O_4$	192	(*3*)
	Stilbene			
10	(*E*)-3,3′-dimethoxy-4,4′-dihydroxy stilbene	$C_{16}H_{16}O_4$	272	(*3*)
	Benzoic acids			
11	Gallic acid	$C_7H_6O_5$	170	(*7*)
12	Methyl gallate trimethyl ether	$C_{11}H_{14}O_5$	226	(*3*)
13	Syringic acid	$C_9H_{10}O_5$	198	(*7*)
14	Protocatechuic acid	$C_7H_6O_4$	154	(*7*)
15	Vanillic acid	$C_8H_8O_4$	168	(*7*)
16	Benzoic acid	$C_7H_6O_2$	122	(*7*)
17	1-O-galloyl-β-D-glucose	$C_{13}H_{16}O_{10}$	332	(*7*)

Continued on next page.

Table 1. (Continued). Chemical constituents identified in maple syrup

Benzoic acids				
18	Resorcylic acid	$C_7H_6O_4$	154	(*7*)
19	Gentisic acid	$C_7H_6O_4$	154	(*7*)
Benzaldehydes				
20	Catechaldehyde	$C_7H_6O_3$	138	(*3*)
21	Vanillin	$C_8H_8O_3$	152	(*6*)
22	Syringaldehyde	$C_9H_{10}O_4$	182	(*6*)
Phenylpropanoids				
23	p-coumaric acid	$C_9H_8O_3$	164	(*7*)
24	Ferulic acid	$C_{10}H_{10}O_4$	194	(*7*)
25	4-methoxycinnamic acid	$C_{10}H_{10}O_3$	178	(*7*)
26	Sinapic acid	$C_{11}H_{12}O_5$	224	(*7*)
27	Chlorogenic acid	$C_{16}H_{18}O_9$	354	(*7*)
28	Syringenin	$C_{11}H_{14}O_4$	210	(*3*)
29	Coniferyl alcohol	$C_{10}H_{12}O_3$	180	(*6*)
30	C-veratroylglycol	$C_{10}H_{12}O_5$	212	(*3*)
31	Dihydroconiferyl alcohol	$C_{10}H_{14}O_3$	182	(*5*)
32	Syringoyl methyl ketone	$C_{11}H_{12}O_5$	224	(*1*)
Flavonoids				
33	Catechin	$C_{16}H_{16}O_5$	288	(*7*)
34	Epicatechin	$C_{16}H_{16}O_5$	288	(*7*)
35	Astragalin	$C_{21}H_{20}O_{11}$	448	(*7*)
36	Kaempferol 3-O-galacotoside	$C_{21}H_{20}O_{11}$	448	(*7*)
37	Isoquercetrin	$C_{21}H_{20}O_{12}$	464	(*7*)
38	Quercitrin	$C_{21}H_{20}O_{11}$	448	(*7*)
39	Rutin	$C_{27}H_{30}O_{16}$	610	(*7*)
Other phenolic compounds				
40	2-Hydroxy-3′,4′-dihydroxyacetophenone	$C_8H_8O_4$	168	(*3*)
41	1-(2,3,4-trihydroxy-5-methylphenyl)-ethanone	$C_9H_{10}O_4$	182	(*3*)
42	2,4,5-Trihydroxyacetophenone	$C_8H_8O_4$	168	(*3*)
43	3′,4′,5′-Trihydroxyacetophenone	$C_8H_8O_4$	168	(*3*)

Continued on next page.

Table 1. (Continued). Chemical constituents identified in maple syrup

Other phenolic compounds				
44	Catechol	$C_6H_6O_2$	110	(*3*)
45	Syringol	$C_8H_{10}O_3$	154	(*1*)
46	Homovanillic acid	$C_9H_{10}O_4$	182	(*6*)
Pyrazines				
47	2,6-Dimethylpyrazine	$C_6H_8N_2$	108	(*1*)
48	Methylpyrazine	$C_5H_6N_2$	94	(*1*)
49	2,5-Dimethylpyrazine	$C_6H_8N_2$	108	(*1*)
50	2,3-Dimethylpyrazine	$C_6H_8N_2$	108	(*1*)
51	2,5-Dimethyl-3,6-diisobutylpyrazine	$C_{14}H_{24}N_2$	220	(*1*)
52	Ethylpyrazine	$C_6H_8N_2$	108	(*1*)
53	2-Ethyl-6-methylpyrazine	$C_7H_{10}N_2$	122	(*1*)
54	2-Butylpyrazine	$C_8H_{12}N_2$	136	(*1*)
55	2,3-Dimethyl-5-isopropylpyrazine	$C_9H_{14}N_2$	150	(*1*)
Other compounds				
56	3-Hydroxy-2-pyrone	$C_5H_4O_3$	112	(*1*)
57	2,3-Dihydro-3,5-dihydroxy-6-methyl-4-pyranone	$C_6H_8O_4$	144	(*1*)
58	2-Methylbenzoquinone	$C_7H_6O_2$	122	(*1*)
59	3-Methylmaleic anhydride	$C_5H_4O_3$	112	(*1*)
60	Homosotolone	$C_5H_4O_3$	142	(*1*)
61	Furaneol	$C_6H_8O_3$	128	(*1*)
62	2-Methyl-2-cyclopentenone	C_6H_8O	96	(*1*)
63	2-Hydroxymethyl-2-cyclopentenone	$C_6H_8O_2$	112	(*1*)
64	Cyclotene	$C_6H_8O_2$	112	(*1*)
65	2-Hydroxymethylcyclopent-2-en-1-ol	$C_6H_{10}O_2$	114	(*1*)
66	Hexanoic acid	$C_6H_{12}O_2$	116	(*1*)
67	2-Ethylhexanoic acid	$C_8H_{16}O_2$	144	(*1*)
68	Acetoin	$C_4H_8O_2$	88	(*1*)
69	Isopropenyl methyl ketone	C_5H_8O	84	(*1*)
Process-derived compounds				
70	Quebecol	$C_{24}H_{26}O_7$	426	(*8*)

Biological Effects of Maple Syrup

Unfortunately, there is a scarity of reports on the biological evaluation of maple sap and syrup extracts. A study showed that phenolic-enriched extracts of maple sap and syrup, collected at different periods in the season, had antioxidant and antimutagenic activities (*11*). The later collection periods of sap results in the darker grades of maple syrup which have correspondingly higher levels of phenolics. These authors found that the total polyphenol concentration (in gallic acid equivalents, GAEs) increased from 16.51-8.51 g GAE/100g to 24.6 g GAE/100g for samples collected later in the season. Further, in that study the authors concluded that the phenolic constituents which are present in maple syrup in their glycosylated forms were more active than their corresponding aglycones.

In a separate study, the antioxidant activity, inhibition of nitric oxide (NO) overproduction in RAW264.7 cells (to measure anti-inflammatory activity), and human cancer cell antiproliferative effects of maple sap and syrup extracts were evaluated (*12*). For that study, maple sap and syrup were collected from 30 producers at different periods of harvest from three different regions of Quebec, Canada. These authors reported that later collections of sap which had more phenolics also exhibited better activity. Overall, the ethyl acetate extracts of these different samples of maple sap and syrup were found to significantly inhibit the lipopolysaccharide-induced NO overproduction in the RAW264.7 murine macrophages. The authors found that the maple syrup extracts were significantly more active than maple sap extracts and concluded that the transformation of maple sap into syrup increases NO inhibition activity. Notably, the highest NO inhibition induced by the maple syrup extracts was observed at the end of the season. In addition, the darker grade of maple syrup was found to be more active than clear maple syrup. Thus, the authors concluded that ‘colored oxidized’ compounds could be responsible for the observed biological activity. Also, the maple syrup extracts showed selective *in vitro* antiproliferative activity against a panel of lung, colon, breast, prostate and brain human cancer cells.

Finally, recent research in our laboratory showed that a maple syrup butanol extract, and several of its individually purified phenolic constituents, have potent antioxidant activities (*3*). Using the diphenylpicrylhydrazyl (DPPH) free radical scavenging assay, the antioxidant activities of the maple syrup compounds **1-9**, **11**, **13**, **20**, **22**, **29**, **30**, **33**, **34**, **40**, **41**, **44** were comparable to vitamin C and the commercial synthetic antioxidant, butylated hydroxytoluene (BHT). The IC_{50} of the antioxidant activities of the different compounds ranged from 20-1400 μM. Compounds **8**, **11**, **20**, **40**, **41** showed superior antioxidant activities when compared to vitamin C (IC_{50} = 58 μM). Among the diverse phenolic subclasses of compounds identified in the maple syrup butanol extract, the general trend in antioxidant activity was flavonols > benzoic acids, benzaldehyde > coumarins > other simple phenolics > phenylpropanoids > stilbene, lignans. Thus all of the above studies (*3*, *11*, *12*), suggest that the phenolic constituents may be responsible in large part for the observed biological effects of maple syrup.

It should be noted that phenolics have been implicated in the prevention of several chronic human diseases mediated by oxidative stress and inflammation (reviewed in (*9*, *10*)). Oxidative stress can cause damage to biomolecules including lipids, proteins, and DNA, resulting in an increased risk for several chronic human diseases. These include inflammatory and cardiovascular diseases, certain cancers, diabetes, Alzheimer's disease, and age-related functional decline. It is possible the wide range of phenolic antioxidants found in maple syrup may help to protect cellular systems from oxidative damage thereby also lowering the risk of certain chronic human diseases. However, while the wide array of chemical constituents, both natural and process-derived, found in maple syrup may impart biological effects and potential health benefits to this natural sweetener, *in vivo* studies to evaluate these properties are needed to confirm this.

Conclusions

Maple syrup is the largest commercially produced and consumed natural product which is obtained entirely from the sap of deciduous trees. During the process of tapping of maple trees, natural phenolic compounds present in the tree sap ultimately ends up (and are concentrated 40X) in maple syrup. In addition, because of the intensive heating conditions required to transform sap to syrup, process-derived compounds are also formed and are present in maple syrup. Thus, maple syrup contains a wide diversity of bioactive chemical constituents in combination with minerals, amino acids, organic acids, phytohormones, peptides and natural sugars. The complex cocktail of maple syrup constituents may impart potential health benefits to maple syrup, as suggested by *in vitro* studies, but more research, particularly *in vivo* studies, would be needed to confirm this. In conclusion, given the wide diversity of bioactive compounds present in maple syrup, evaluation of the health benefits of this natural sweetener holds great promise.

Acknowledgments

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Chapter 19

Sesame Seed (*Sesamum indicum* L.) Extracts and Their Anti-Inflammatory Effect

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Steroids and non-steroidal anti-inflammatory drugs (NSAIDs) are the main choices for controlling inflammation. However, their adverse effects limit their clinical use. Sesame (*Sesamum indicum* L.) is one of the oldest cultivated plants in the world. Its extracts, such as sesame oil, have been widely used since ancient times. Recently, sesame oil and its lignan sesamol have been proved to be potent anti-inflammatory agents. They have an excellent protective effect against endotoxin-associated inflammatory damage because they inhibit the release of inflammatory mediators. Sesamol also inhibits endotoxins from binding to its receptor; this reduces inflammatory transcription factor NF-κB activation. In summary, sesame oil or sesamol may be beneficial for reducing the inflammatory response in inflammation-associated diseases.

Introduction

Sesame seed is one of the most-used ingredients in Asian dishes in China, India, Japan, and Taiwan. Its oil is high in polyunsaturated fats and in the unique antioxidants sesamol and sesamin. Sesame seed oil and other extracts are believed to have antibacterial and anti-inflammatory properties (*1*). Therefore, it is often recommended in alternative medicine. Despite a growing number of recent studies showing sesame seed oil's anti-inflammatory property, the U.S. Food and Drug Administration has not approved it as a drug for controlling inflammation.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Botanical Descriptions

Sesame (*Sesamum indicum*) is an annual bushy plant. The prime season for sesame seeds is between September and April. Sesame can grow as high as seven feet tall, though most plants range between two and four feet. The leaves are oval, deeply-ribbed, light green, and 7.5 to 12.5 cm long. The gamopetalous flowers, which look somewhat like foxglove, are white to purple, tubular, 3 to 5 cm long, have a four-lobed mouth, and produce pods inside measuring 2.5-8.0 cm long by 0.5-2.0 cm in diameter (*2*). When ripe, the pods burst and release small, flat, oval seeds about 1 mm thickness. The seeds are high in oil and, come in a variety of colors depending on the plant variety: shades of brown, red, black, yellow, and, most commonly, a pale grayish ivory (*3*).

Cultivation and Use

Sesame is an ancient oilseed. There is botanical and textual evidence for sesame cultivation in the ancient world. Excavations at the Indus Civilization site in Harappa (contemporary West Punjab, Pakistan) have yielded charred sesame from a stratum attributed to 3050-3500 B.C. Sesame was grown during the ancient Harappan, Mesopotamian, and Anatolian eras for its edible seed and its oil (*4*). Ancient peoples used sesame seed oil as a food, salve, medication—especially in Chinese and Indian herbal medicine—and source of light (*3*). As early as the 8th century B.C., the Chinese used sesame seeds to treat insect bites and toothache. Indians believed that sesame seed oil gave the body energy (*4*). Nowadays, sesame is grown in many areas of the world. India ranks first in area (46.5%) under sesame cultivation; it produces about 27.9% of the world's sesame (*5*), but the crop is also grown in China, Burma, Sudan, Ethiopia, and other places. U.S. commercial production reportedly began in the 1950s, primarily in Texas and the southwestern states; however, the U.S. imports more sesame than it grows. Sesame seed oil is nutritious and has great moisturizing, soothing, and emollient qualities. Therefore, sesame seed oil is currently used in salad and as a cooking oil, an ingredient in cosmetics, and in the manufacture of soaps, pharmaceuticals, and lubricants (*3*).

Sesame Extracts for Preventing Inflammatory Diseases

Inflammation

Burns, chemical irritants, frostbite, toxins, infection by pathogens, some physical injuries, ischemia, oxidative stress, and immune reactions caused by hypersensitivity induce inflammatory responses. During these responses, inflammatory cells (macrophages and neutrophils) and additional effector molecules (cytokines and chemokines) are delivered to the stimulated sites to accelerate the elimination of harmful proinflammatory agents. In addition, the inflammatory response is associated with the preventing the spread of infection and promoting the repair of injured tissue (*6*). The release of inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and nitric oxide (NO), not only activates inflammatory cells, but also changes blood vessel permeability.

Therefore, inflammatory responses are characterized by pain, redness, heat, and swelling at the inflammatory site (*6*). Although inflammation is the body's defense response, inducing an inappropriate or excess inflammatory response leads to severe fever, agonizing pain, and even organ damage. Rheumatoid arthritis, shoulder tendinitis, gouty arthritis, hay fever, asthma, allergies, and autoimmune disorders are all associated with inflammation. In particular, sepsis, a systemic inflammatory response caused by bacterial endotoxins, is the primary cause of death in patients in intensive care units (*7*). Severe sepsis causes acute respiratory distress syndrome, multiple organ dysfunction, and death (*8*). Mortality is 26% in patients with systemic inflammation (*9*). However, effective therapeutic interventions for treating patients with sepsis are being developed.

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Currently Used Anti-Inflammatory Drugs

To control inflammatory diseases, anti-inflammatory drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) (aspirin and diclofenac, for example), corticosteroids (such as prednisone), methotrexate, and leflunomide, have been used. However, they all have significant adverse side effects.

NSAIDs are commonly used in clinical practice to manage pain, fever, and various inflammatory diseases in patients; however, NSAIDs have been identified as gastrotoxic (*10*, *11*). Mild to severe gastric ulceration and bleeding occur not only in patients who undergo long-term NSAID treatment, but also in patients who intentionally or accidently overdose on NSAIDs (*12*, *13*). Acute gastrointestinal symptoms can even be found within 2 hours after an NSAID overdose (*13*). Although short-term corticosteroid treatment rarely induces serious adverse side effects, long-term treatment may lower the body's ability to fight off infections or may make infections harder to treat. Excessive exposure to corticosteroids may cause Cushing's syndrome (*14*). Methotrexate is commonly a first-choice disease-modifying antirheumatic drug. However, methotrexate may cause blood dyscrasias (some fatal) and liver cirrhosis (*15*). The adverse effects of leflunomide, which is used to treat rheumatoid arthritis, include diarrhea, alopecia, increased liver enzyme expression, and hepatic failure (*15*).

Anti-Inflammatory Effect of Sesame Seed Oil

The primary constituents of sesame seed oil, which is derived from sesame seeds, include fatty acids and lignans. The fatty acids are palmitic acid (16:0; 7.0-12.0%), palmitoleic acid (16:1; less than 0.5%), stearic acid (18:0; 3.5-6.0%), oleic acid (18:1; 35-50%), linoleic acid (18:2; 35-50%), linolenic acid (18:3; less than 1%), and eicosenoic acid (20:1; less than 1%). The nonfat portion (1-2 wt%) contains lignans such as sesamin, sesamol, sesamolin, sesaminol, and episesamin. Sesame seed oil is unique because of its unusually high oxidative stability and anti-inflammatory property compared with other edible oils (*16*). Sesame seed oil's strong antioxidant activity has been attributed mainly to γ-tocopherol and antioxidative sesame lignans such as sesamin, sesamolin, and sesamol (*17*) (Figure 1).

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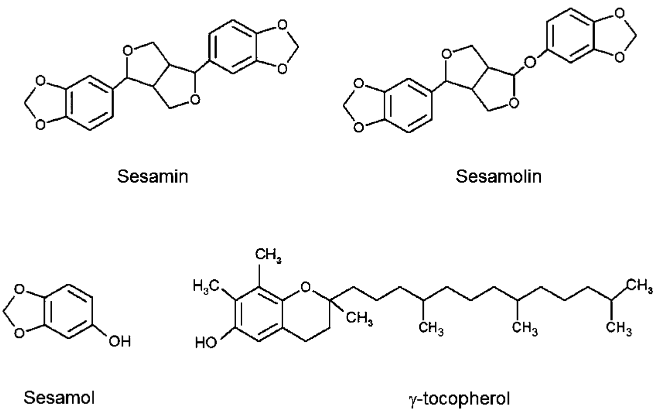

Figure 1. The major chemical ingredients of sesame seed extract.

Lipopolysaccharide (LPS), the important component of the Gram-negative bacterial cell wall, is a potent stimulator of the inflammatory response. In animals and humans, LPS intoxication leads to a systemic inflammatory response and causes the dysfunction of various organs, including lungs, liver, and kidneys (*18*). Because LPS is considered the primary clinical cause of the systemic inflammatory response (*19*), it is usually used in animal models of sepsis and inflammation. LPS-induced proinflammatory cytokines, such as TNF-α, IL-1β, and NO, are involved in generating oxidative stress and organ damage in LPS intoxication (*8*).

Sesame seed oil has an excellent anti-inflammatory effect in LPS-induced systemic inflammatory models and lead-plus-LPS-induced hepatic inflammation. Sesame seed oil reduces the release of the proinflammatory mediators NO, TNF-α, and IL-1β in serum in LPS-stimulated rats (*20*) and in the liver in rats treated with LPS-plus-lead (*21*). Both of these anti-inflammatory effects are associated with oxidative stress inhibition and organ protection in LPS-treated rats.

Anti-Inflammatory Effect of Sesamol, a Lignan in Sesame Seed

Sesamol (3, 4-Methylenedioxyphenol) ($C_7H_6O_3$), one of the sesame seed oil lignans, has been generally regarded as the main antioxidative component in sesame seeds (*22*). During sesame seed oil manufacturing, sesamolin can be converted to other lignans, including sesamol, sesaminol, and sesamol dimers.

Some details of the mechanism by which LPS activates phagocytes are now understood. After bacteria release LPS into the bloodstream, it first binds to LPS binding protein (LBP), an acute-phase reactant in the blood. LBP catalyses the transfer of LPS to CD14 (an LPS receptor), which increases the LPS-induced activation of monocytes, macrophages, and polymorphonuclear neutrophils 100-1000 times (*23*). LPS·CD14 complexes activate LPS signaling receptor MD2 and toll like receptor 4 (TLR4) (*24*). When activated, TLR4 recruits adapter molecules within the cytoplasm of cells to propagate a signal. The adapters activate the downstream protein kinases (IRAK1, IRAK4, TBK1, and IKKi) that amplify the signal, which results in the activation of MAPK and IKK. This activation leads, in turn, to the activation of the transcription factor nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), both of which mediate the production of inflammatory cytokines (*25*). NF-κB is a fundamental transcription factor vital for the expression of proinflammatory genes (*26*). Non-activated NF-κB is retained in the cytoplasm by binding to an inhibitory protein, inhibitor κB (IκB)-α. During inflammation, LPS activates the NF-κB pathway and phosphorylates IκB, which leads to a disassociation of the IκB/NF-κB complex. Subsequently, free NF-κB enters the nucleus and binds to the DNA, thereby allowing rapid gene activation and the expression of TLR4-associated protein and proinflammatory mediators (*27*).

Sesamol inhibits LPS receptor activation in macrophages. Sesamol causes macrophages to downregulate the LPS-induced proinflammatory mediators TNF-α, IL-1β, and NO (*28*), inhibits the LPS-receptor (TLR4) activating pathway in macrophages, and inhibits bound LPS and LBP (IC_{50} = 0.016 ± 0.003 nM). It is more effective than polymyxin B (IC_{50} = 126 ± 28 nM), a well-known and potent antibiotic that binds and neutralizes bacterial LPS. Sesamol inhibits LPS from binding to LBP even after LPS and LBP have been co-incubated (*29*). The IC_{50} of sesamol in the displacement effect is (1.3 ± 0.2 μM). In addition, sesamol inhibits NF-κB activation downstream of the TLR4-activating pathway. It also inhibits LPS-induced IκB phosphorylation and NF-κB translocation without affecting macrophage viability (*28*). It is likely that sesamol blocks LPS from binding to LPS binding protein and inhibits the inflammatory response.

Adverse Effects and Reactions

In sesame's long history as a crop, no harmful effects of sesame seed extracts in humans have been reported. However, some studies report that sesamol is carcinogenic in rodents.

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Chapter 20

Functional Food Components for Preventing and Combating Type 2 Diabetes

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Increased intake of refined high calorie foods globally is leading to disorders of carbohydrate metabolism resulting into chronic disease such as obesity associated type 2 diabetes. Important therapeutic approaches available for managing early stages of type 2 diabetes is by controlling the absorption of glucose through the reduction of starch hydrolysis by inhibiting pancreatic alpha-amylase and limiting the absorption of glucose by inhibiting intestinal alpha-glucosidase. Natural alpha-amylase and alpha-glucosidase inhibitors from plant foods offer a good strategy to control the post-prandial hyperglycemia and provide benefits without side effects. Further these plant foods have bioactive profiles that have potential to manage the macrovascular complications such as blood pressure through inhibition of Angiotensin I-Converting Enzyme (ACE) and micro vascular complications through antioxidant activity. These biochemical strategies provide the structure-function rationale for animal and clinical studies for designing effective dietary therapies for managing type 2 diabetes.

Changing Dietary Pattern: Impact on Global Diseases

The interaction of genetics and the environment is the foundation for the evolution of human health and diseases. Changes in dietary pattern and in nutritional composition have had significant evolutionary impact in the past and they now affect the health of contemporary human populations (*1*). The evolution

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of human dietary pattern has been mostly driven by the necessity, availability, taste, socio-cultural interactions and cost of the diet. With the development and advancement of agriculture since 10,000 years ago, humans have gradually shifted from a diverse plant-based diet that provided essential vitamins, minerals and over 25,000 phytochemicals to a diet based on refined grains, added oils, sugar and salt (*2*). During last century, specifically in post World War II, carefully designed foods on the basis of taste, cost and convenience was promoted in the human diet worldwide. The development of this modern packaged and fast food often disregarded or neglected a wider and more comprehensive nutritional and health value of our food system (*3*). The marketing of popular and profitable manufactured foods along with globalization of economies and agriculture threatens to homogenize diet and dietary pattern in modern times. Such changes in human diet and changes in lifestyle have significantly impacted in the rapid global emergence of non-communicable chronic diseases (NCD) like cardiovascular disease, diabetes and cancer (*4*).

The modern nutritional science developed on the basis of the discovery of specific dietary insufficiencies as the cause of human disease and malfunction (*5*). But during last two decades more studies have focused on the development of dietary guidelines to combat chronic diseases like obesity-linked diabetes, cardiovascular disease and cancer. Over the past 40 years international studies comprising controlled trials of risk factors and prospective cohort study of disease end points have documented the lack of diversity of human dietary pattern and its association with increased risk of chronic diseases (*6*). To counter this fruits, vegetables, whole grains, and nuts are generally associated with lower risk of chronic diseases. Whereas processed refined foods with high calories from saturated fats and carbohydrates and sugar sweetened beverages increase the risk of chronic diseases. Therefore the impact of food and dietary pattern on human health largely depends on the complex, synergistic contributions and interactions among food structure, preparation methods, fatty acid profile, carbohydrate quality (glycemic index, fiber content), protein type, micronutrients and phytochemicals (*5*). Healthy diet mostly emphasizes whole or minimally processed foods and vegetable oils, with few highly processed refined foods or sugary beverages. The characteristics of such healthy foods are low in salt, *trans* fat, saturated fat, refined carbohydrates, added sugar and high in unsaturated fats, fiber, antioxidant, minerals and phytochemicals.

Type 2 Diabetes: An Emerging Pandemic

The incidence of diabetes and cardiovascular diseases are increasing in an alarming rate and becoming a major threat to human health in the 21st century. Cardiovascular diseases, diabetes, obesity, cancer and respiratory conditions account for 59% of the 56.5 million deaths annually and 45.9% of the global burden of disease (2009) (*7*). Diabetes particularly type 2 diabetes is one of the most common chronic diseases in nearly all countries and continues to increase in numbers and significance worldwide. Earlier type 2 diabetes was regarded as a health risk associated with affluence but profound changes in the quality,

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quantity and source of food consumed in many developing countries, combined with a decrease in levels of physical activity and changing lifestyle have led to an increase in the prevalence of this disease irrespective of economic background and race. The cause of this global epidemic are complex and include political, demographic, environmental, genetic and lifestyle factors (*8*).

According to estimates of the World Health Organization and the International Diabetes Federation, the prevalence of diabetes has increased from 100-135 million affected adults worldwide in 1994-1995 to approximately 285 million adults in 2010 with more than 90% of cases considered type 2 diabetes (*9*). World Health Organization projected that by the year 2030 the number of prevalent patients with type 2 diabetes in the world will increase by 7.7% and will reach about 439 million while diabetes deaths will be double (*10*). In developed countries the majority of diabetes patients are aged over 60 years, whereas most of the diabetes patients in the developing countries are of working age between 40 and 60 years. Around 1.1 million people died due to diabetes in 2005 and actual number is likely to be much higher as cause of death often diagnosed as heart disease or kidney failure. Diabetes patients in the North America and Caribbean region is around 37.4 million (2010) and type 2 diabetes has been considered as a new epidemic in the American pediatric population (*11*, *12*). Population growth, ageing of population and urbanizations are key factors which will lead to a 54% increase in numbers with diabetes by 2030 (*9*). Globally, India leads the number of diabetes patients with a current figure of 50.8 millions followed by China (43.2 millions) and USA. Out of the anticipated absolute global increase of 154 million people with diabetes, India and China alone will account for thirty-six percent (*9*). Rapid urbanization and economic growth of these countries slowly inducing unhealthy diet, bad lifestyle and psychological stress which are the key contributors for the outburst of non-communicable chronic diseases including type 2 diabetes (*13*).

The global emergence of obesity and diabetes is also an economic issue as it is a health issue. Diabetes along with cardiovascular disease has a significant socio-economic impact on individuals, families, health system and countries (*8*). Several economic factors like expanding labor market opportunities for women, increased consumption of food away from home, rising costs of healthy foods relative to unhealthy foods, growing quantity of caloric intake with declining overall food prices and decreased requirements of occupational physical activity have significantly contributed in the prevalence of diabetes particularly in developing countries (*14*, *15*). Obesity and diabetes impose a considerable economic burden on societies. World health organization reported that during 2006-2015, China will lose $558 billion in foregone national income due to heart disease, stroke and diabetes alone (*10*). In the Latin America one out of every three hospital bed-days are occupied for diabetes related cause, with average costs for a year of diabetes care at roughly $550 per person, exceeding most per capita gross domestic product health expenditures (*16*). Large portion of this expenditures cover hospital admission and medicine but sometime exclude diabetes related long term complications such as heart disease, stroke, blindness, renal failure and limb amputations (*16*).

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The rapid changes in the incidence of type 2 diabetes in last two decades have mostly governed by the lifestyle and environmental factors rather than the genetic cause (*17*). Overweight and obesity is one major contributor in the onset of type 2 diabetes. Several studies suggested that environmental factors present at birth like mother's body mass index and child's birth weight, growth trajectories of children in the earliest years of life, subsequent eating and activity patterns, and behavior in childhood strongly relate to the trends in diabetes (*18*). All factors which promote obesity like consumption of excess energy, particularly increases in the intake of saturated fatty acids, sugar-sweetened beverages and starchy foods, and the consumption of less fiber with sedentary lifestyle are responsible for the global outbreak of type 2 diabetes (*13*). Type 2 diabetes and cardiovascular disease have many common risk factors and they are highly correlated with one another. "Metabolic syndrome" which is a powerful determinant of diabetes and cardiovascular disease includes hypertension, dyslipidemia, insulin resistance, hyperinsulinemia, glucose intolerance and obesity (particularly central obesity) (*19*).

Type 2 diabetes mellitus is a chronic metabolic disorder characterized by relative insulin deficiency due to impaired insulin production combined with peripheral insulin resistance (*17*). The primary cause of fasting hyperglycemia is due to an elevated rate of basal hepatic glucose production in the presence of hyperinsulinemia; whereas postprandial (after a meal) hyperglycemia is due to the impaired suppression of hepatic glucose production by insulin and decreased insulin-mediated glucose uptake by muscle (*20*). Patients with chronic diabetes experience different metabolic and physiological disorder. Significant morbidity and mortality due to both microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (heart attacks, stroke, and peripheral vascular disease) complications are common in most of the diabetic patients (*21*). Type 2 diabetes is the leading cause of blindness and end stage renal failure in the United States. The risk of heart disease and stroke are two to four times more frequent in person with diabetes and 50% of people with diabetes die due to cardiovascular disease (*21*).

Most cardiovascular risk factors in diabetic patients are directly affected by an acute increase of glycemia and are modified in the postprandial phase (*22*). Obesity and diabetes are pro-inflammatory states in which inflammatory mechanisms could contribute to insulin resistance. Many studies reported that atherosclerosis is an inflammatory disease even in diabetes (*23*). All major risk factors for type 2 diabetes generally induce local or systemic low-grade inflammation and chronic low-grade inflammation will eventually lead to over-diabetes if counter-regulation of inflammation and metabolic stress are compromised because of a genetic or epigenetic predisposition (*22*). Cellular oxidation and production of free radicals are common mechanisms through which hyperglycemia exerts the harmful effect on the body (*24*).

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Oxidative Stress and Cellular Homeostasis

Reactive oxygen species (ROS) and associated free radicals associated with unpaired electrons of atoms react with various molecules at the site of formation or induce damaging chain reactions from initial formation (*25*). They are produced by multiple pathways and their production in cellular systems is balanced by enzymatic and non-enzymatic antioxidant systems. ROS are essential for many cellular functions and play significant role in biochemical processes, including cellular differentiations, growth arrestment, apoptosis, immunity and defense against microorganisms and intracellular messaging (*26*). ROS have a significant role in normal metabolism and perturbation of pro-oxidant/antioxidant balance can lead to a toxic state in which macromolecules (lipids, proteins or DNA) are oxidatively damaged and cellular function is altered. This cellular phenomenon in part contributes to “oxidative stress” (*25*).

Oxidative stress plays an important role in the pathogenesis of vascular alterations by either triggering or exacerbating the biochemical processes accompanying the metabolic syndrome (*27*). The prevalence of hypertension in diabetes may be linked to the increased generation of free radicals in cell (*28*). Many studies have shown that reduction of antioxidant defense is an important cardiovascular risk factor and increased free radical generation is present in diabetes (*29–31*). Oxidative stress may be amplified by a concomitant antioxidant deficiency that may favor the propagation of oxidative alterations from intra to extracellular spaces and from confined to distant sites, thus realizing a systemic oxidative stress state. Overnutrition, saturated fatty acids and obesity play key role in the excessive production of ROS and chronic overnutrition is associated with insulin resistance (*25*). ROS when produced in excess, significantly influence insulin signal transduction and insulin resistance (*24*).

Multiple pathways are involved leading to insulin resistance and alteration of several pathways is required before it becomes clinically evident (*30*). Study comparing four different models of insulin resistance showed a direct correlation between mitochondrial oxidative stress and insulin resistance in all models (*32*). Mitochondrial oxidative stress could be a unifying element of insulin resistance, acting principally as a nutrient sensor in key metabolic pathways in tissues to regulate nutrient intake in accordance with the energy supply (*25*). Obesity-associated oxidative stress also includes multiple processes and they are: i) a high metabolic load that exposes cells to an overload of nutrients with excessive mitochondrial oxidation and enhanced ROS generation, ii) inflammatory states associated with obesity, iii) endoplasmic reticulum stress and iv) endocrine dysregulation such as high levels of insulin (*25*). Anderson et al. (*33*) observed that increased fat intake is capable of shifting the cellular redox environment to a more oxidized state in the skeletal muscle of both rodents and humans regardless of weight gain. Other studies showed that NADPH oxidase is also involved in the higher generation of ROS in response to saturated fatty acids in muscle cells (*34*). High consumption of fat and chronic exposure to fatty acid are also associated with increased ROS production, both in β-cells, leading to dysfunction and cell death, and in the liver, leading to hepatic insulin resistance (*35*).

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Acute hyperglycemia works through the production of an oxidative and nitrosative stress (*31*). Induction of oxidative stress in hyperglycemia involves several mechanisms including glucose auto-oxidation, advanced glycation end product formation, abnormal arachidonic acid metabolism and its coupling to cyclooxygenase catalysis, protein kinase C activation, increase in the activity of nitric oxide (NO) synthase and activation of the aldose reductase pathway (*30*). Hyperglycemia induces an overproduction of superoxide by the mitochondrial electron transport chain. Overproduction of superoxide is generally accompanied by the increased NO generation which favors the formation of strong oxidants peroxynitrite and subsequent DNA damages (*31*). DNA damage stimulates the activation of the nuclear enzyme, poly(ADP-ribose) polymerase which in turn depletes the intracellular concentration of its substrate NAD^+, slowing the rate of glycolysis, electron transport and ATP formation and produces an ADP ribosylation of the GAPDH (Glyceraldehyde-3-phosphate dehydrogenase). Ultimately these processes result in an acute endothelial dysfunction in diabetic blood vessels and significantly contribute to the development of cardiovascular disease (*30*, *31*).

Many studies have suggested that postprandial hypertriglyceridemia and hyperglycemia independently induce endothelial dysfunction through oxidative stress (*36*). In diabetic patients low density lipoproteins (LDL) are more prone to oxidation in postprandial phase. Oxidized LDL activates endothelial cells with the promotion of an immune response and leads to the formation of lipid-laden macrophages (*24*). These endothelial damages are generally favored by insulin resistance and obesity. In general under normal condition reduced state of LDL is maintained by vitamin E, which also acts by regulating inflammatory reactions and metabolic pathways, including platelet aggregation. The prevention of cardiovascular events is associated with consumption of vitamin E and C as they reduce LDL oxidation through synergistic action (*37*). All these evidences suggest that hyperglycemia and obesity acutely induce alterations of the normal human homeostasis.

Many studies evaluating role of nutritional factors on metabolic syndrome suggested that caloric restriction may be associated with life prolongation probably through an improvement of the cell redox balance (*38*). Nutritional perturbation increase mitochondrial ROS generation and significantly influences the oxidative damage of the cell. Many *in vitro* and *in vivo* studies have shown that dietary antioxidants, taken either as extracts or part of a food itself have beneficial effects on glucose metabolism (*39–42*). Other than food, gut microbiota also play significant role in the regulation of host energy homeostasis and adiposity. Most of the strategies to counter oxidative stress in human focused on the enhancement of ROS removal either through antioxidants or drugs that enhance endogenous antioxidant defense (*43*). An alternate approach to reduce oxidative stress is inhibiting ROS production by blocking enzymes involved it its synthesis. Diet along with physical exercise is crucial to maintain cellular redox balance and thus play significant role to combat oxidative stress induced diseases including type 2 diabetes (*24*).

Diet as a Preventive Measure to Combat Type 2 Diabetes

Maintenance of glucose homeostasis is very important for diabetic patients as failure of this strict hormonal balance can result in a multi-symptom disorder and can lead to obesity, hyperglycemia, impaired glucose tolerance, hypertension and dyslipidemia (*44*). Healthy diet and proper nutritional compositions are key factors in the regulation of glucose metabolism. Diet or dietary treatment is able to avoid the rapid rises and falls of plasma glucose levels caused by meal consumption, and is effective in controlling hyperinsulinaemia, dyslipidemia and other cardiovascular risk factors (*35*). Worldwide there are many dietary patterns and some of which promotes health and combat diseases while others increase the risk of chronic diseases. Other than dietary pattern, several dietary components are also critical in controlling chronic diseases. Daily consumption of high glycemic index foods like white bread, rice, pasta, cornflakes, and ice-cream may increase the risk of obesity, type 2 diabetes and cardiovascular disease by promoting excessive calorie intake, pancreatic β cell dysfunction, dyslipidemia and endothelial dysfunction (*45*). Among different dietary pattern Mediterranean diet is helpful to lower the risk of coronary heart disease, diabetes, cancer and cognitive impairment (*46*). The role of Mediterranean diet against diabetes includes a high intake of fiber low intake of *trans* fatty acids and moderate intake of alcohol (*47*). Mediterranean diet in general rich in non-unsaturated fatty acids and thus improves lipid profile and glycemic control in people with diabetes. Consumption of foods like fruits, vegetables, whole grains and beans rich in soluble and insoluble fiber has been suggested to reduce the risk of coronary heart disease, diabetes and cancer (*48*, *49*).

Role of Fiber

Dietary fiber is classified as water soluble and water insoluble fiber. Foods such as fruits, oats, barley, eggplant, oakra and legumes are rich source of soluble fibers like pectins, gums and mucilages (*50*). Water insoluble fibers like lignins and cellulose are more common in wheat bran, whole grain cereals, and vegetables such as cabbage, carrots and Brussels sprouts (*50*). Soluble fibers are generally fermented to a greater extent than insoluble cellulose. Insoluble forms of fiber generally promotes colonic mucosal health, by increasing fecal bulking and reducing transit time through the gut, where soluble dietary fiber is more influential at metabolic level (*51*). Soluble and insoluble fibers both have significant beneficiary impact on human health but the ideal type and proportion for consumption is not clearly defined. Diets rich in soluble and insoluble fiber have different health benefits like inducing satiety, reducing total energy intake and adiposity, improving glycemic control, reducing blood lipids, and preventing constipation, diverticuli, and other disorder of gastrointestinal tract (*52*). Many studies have been shown that dietary fiber improves glucose metabolism in type 2 diabetes patients (*53*, *54*). High fiber diet generally lower values for fasting and postprandial plasma glucose, hemoglobin A_{1C}, total cholesterol including low density lipoprotein (LDL), and triglycerides (*50*, *55*).

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Dietary fiber particularly soluble fiber improves glucose control and significantly reduces postprandial plasma glucose levels and the mean daily blood glucose profile (*50*). Several cohort studies reported that cereal fiber such as wheat bran, which is mostly insoluble fiber markedly reduce the risk of diabetes (*56*). Main characteristics which govern the efficacy of fiber are its quantity, viscosity and rheological properties. Soluble dietary fiber alters food texture, structure and viscosity and thus changes the rate of starch degradation and digestion (*57*). In the gastrointestinal tract viscous gel or hydrated fiber reduces the rate of nutritional absorption by increasing the unstirred layer in the small intestine and thus reduces postprandial glycemia and insulinemia. Giacco et al. (*58*) reported that acute consumption of high fiber meal (legumes, vegetables and fruits) containing 15 g of water soluble fiber reduced postprandial plasma glucose concentrations by 60%, plasma insulin level by 30% and plasma triglyceride levels by 60% in type 2 diabetic patients. Another study have shown that the addition of fiber to a meal or the use of fiber-fortified foods reduce postprandial glycemia possibly through increasing food or meal viscosity (*59*).

Fiber itself does not have any glycemic index as it does not contain any available carbohydrate but the addition of fiber to carbohydrate foods can lower the glycemic index of the food (*50*). Increased fiber intake have been found most important factor for the reduction in circulating levels of C-reactive protein (CRP) and IL-6 (*60*). Six out of seven dietary intervention trials reported that daily consumption of 3-8 g fiber per megajoule led to significant reduction in plasma CRP concentrations by 25-45% (*61*). Several viscous fiber particularly soluble fibers improves plasma cholesterol metabolism by significantly reducing total cholesterol and LDL. Different fiber rich foods such as legumes, oat and barley or fiber from purified sources such as guar gum, psyllium, pectin, konjac mannan, and xanthum gum reduce mortality and morbidity due to cardiovascular diseases (*62*). Mechanisms through which viscous fiber alter plasma lipid concentrations are reduced emulsification of dietary lipids, decreased cholesterol absorption, alterations of bile acid metabolism and hepatic cholesterol synthesis and increased excretion of neutral steroids and bile acids (*63*). National Cholesterol Education Program (NCEP) III report suggested that an increase in viscous fiber of 5 to 10 g/d is associated with an approximate 5% reduction in LDL cholesterol (*64*). Vuksan et al. (*65*) have shown that compared with wheat bran, consumption of viscous fiber blend reduced relative cardiovascular risk by 17% in individuals with type 2 diabetes, by 26% in subjects with the metabolic syndrome and by 28% in healthy subjects. Effects of fiber on satiety comprises gastric distension, slower absorption of macronutrients resulting in a reduction in postprandial glycemia, and enhanced effects of hunger related hormones such as cholecystokinin, glucagon like peptide-1 and peptide YY (*66*). All of the above evidence suggests that dietary fiber in diabetic patients decreases postprandial plasma glucose, insulin and triglyceride concentrations and thus improve the management of type 2 diabetes (*50*, *67*).

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Role of Plant-Based Phenolics or Polyphenols

Phytochemicals are structurally diverse bioactive compounds found in fruits, vegetables, grains and other plant based foods. There are more than 45,000 phytochemicals and they are classified into four major groups including tarpenoids, phenolics and polyphenolics, nitrogen containing alkaloid and sulfur containing compounds (*7*). Phytochemicals are secondary metabolites of plants and provide unique survival and adaptive strategies against biotic and abiotic stresses. They also provide beneficial impact on human health when consumed in the diet. Phytochemicals possess preventative effect against specific diseases, mainly at very early stages of disease developments. Among phytochemicals polyphenol possess anti-inflammatory, antioxidative, chemopreventive and neuroprotective activities and thus associated with lowered risk of major chronic diseases including diabetes, cardiovascular diseases and cancer (*44*). Most of the dietary polyphenols are metabolized by colonic microbiota before absorption. Hydrolysis, ring-cleavage, reduction, decarboxylation and demethylation are different mechanisms through which gut bacteria modulate polyphenols (*68*). The bilogical activity of polyphenols largely depends on the synergistic action and is affected by other constituents present in the diet as well as endogenous factors (*69*).

Polyphenols are virtually present in all plant foods but their levels vary significantly among diets depending on the type and quantity of plant-based food source. Phytochemicals also possess significant antioxidative properties and can protect cell constituents against oxidative damage through direct scavenging of free radicals. Cells responds to phytochemicals mainly through direct interactions with receptors or enzymes involved in signal transduction, or through modifying gene expressions which may results in modification of the redox status of the cell that may trigger a series of redox-dependent reactions (*70*). Dietary polyphenols from different plant based sources influence glucose metabolism by several mechanisms, such as inhibition of carbohydrate digestion and glucose absorption in the intestine, stimulation of insulin secretion from the pancreatic β-cells, modulation of glucose release from liver, activation of insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulation of hepatic glucose output (Figure 1; (*44*)).

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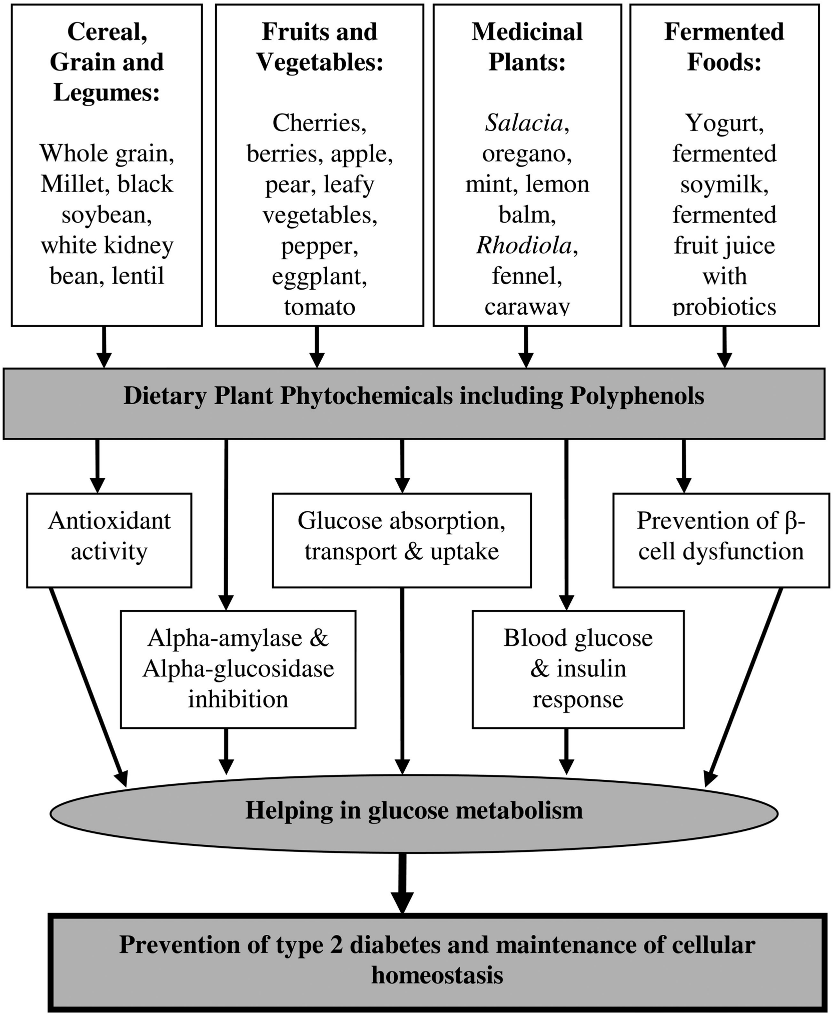
Cereal, Grain and Legumes:
Whole grain, Millet, black soybean, white kidney bean, lentil
Fruits and Vegetables:
Cherries, berries, apple, pear, leafy vegetables, pepper, eggplant, tomato
Medicinal Plants:
Salacia, oregano, mint, lemon balm, *Rhodiola*, fennel, caraway
Fermented Foods:
Yogurt, fermented soymilk, fermented fruit juice with probiotics
Dietary Plant Phytochemicals including Polyphenols
Antioxidant activity
Glucose absorption, transport & uptake
Prevention of β-cell dysfunction
Alpha-amylase & Alpha-glucosidase inhibition
Blood glucose & insulin response
Helping in glucose metabolism
Prevention of type 2 diabetes and maintenance of cellular homeostasis

Figure 1. Source of plant phytochemicals and their role in glucose metabolism and type 2 diabetes prevention.

Carbohydrate digestion and glucose absorption are obvious targets for better glycemia control after high carbohydrate meals and α-amylase and α-glucosidase are the key enzymes responsible for the digestion of dietary carbohydrates to glucose. Inhibition of these digestive enzymes could reduce the rate of glucose release and absorption in the small intestine and consequently suppress postprandial hyperglycemia (*44*). Many *in vitro* studies have been reported that polyphenols include flavonoids (anthocyanins, catechins, flavanones, flavonols, flavones and isoflavones), phenolic acids and tannins (proanthocyanindins and ellagitanins) inhibit α-amylase and α-glucosidase activities (*44*). Polyphenolic extracts of foods including berries (strawberries, raspberries, blueberries and black currants), vegetables (pumpkin, beans and eggplants), colored grains such

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as black rice, green and black tea and red wine also showed α-amylase and α-glucosidase inhibition in *in vitro* studies (*71–79*).

Polyphenols also influence glucose transporters and thus mediate intestinal absorption of glucose. Several flavonoids including chlorogenic, ferulic, caffeic, tannic acids, quercetin monoglucosides, tea catechins and naringenin have been found to inhibit Na^+-dependent SGLT1-mediated glucose transport (*80–83*). Several studies conducted in animal models also showed that polyphenols could alter postprandial blood glucose response. Song et al. (*84*) observed that hyperglycemia was significantly decreased in diabetic rats after administration of glucose with quercetin. In another study, Hanamura et al. (*85*) reported a reduction of plasma glucose level in mice after administration of maltose with crude Acerola polyphenol fraction, suggesting inhibition of α-glucosidase activity and intestinal glucose transport. Berries, a rich source of anthocyanins significantly decreased the peak glucose increment through reducing the rate of sucrose digestion or absorption from the gastrointestinal tract (*45*). Soybean isoflavonoids have shown positive impact on β-cell function in some recent studies (*44*). Choi et al. (*86*) found that isoflavonoids like genistein and daidzein preserved insulin production by β-cell in mice. Flavonoids like quercetin, luteolin and apigenin also showed protective function against β-cell (*87*). Dietary polyphenols may also influence gluocse metabolism through stimulation of peripheral glucose uptake in both insulin-sensitive and insulin-nonsensitive tissues (*88*). Regulation in the expression of genes involved in glucose uptake and insulin signaling pathways was altered through green tea polyphenolic extract in the muscle tissue of metabolic syndrome induced rats (*89*).

Not only glucose metabolism, flavonoid intake also is able to lower the risk of coronary disease and cancer (*90*). High intake of flavonoid (approximately 30 mg/day) was found to be associated with a 50% reduction in coronary heart disease mortality rate (*91*). The mechanism of action of flavonoid against cardiovascular diseases include inhibition of LDL oxidation and inhibition of platelet aggregation and adhesion (*92*, *93*). Polyphenols are also capable of inhibiting cholesterol esterification and intestinal lipoprotein secretion (*94*). Due to the high antioxidant activity of polyphenols it can regulate cellular function through inhibition of pro-oxidant enzymes, induction of antioxidant enzymes and inhibition of the redox-sensitive transcription factors (*95*). These studies along with other epidemiological studies suggest that polyphenols can have a therapeutic role with health-protective benefits by acting as modifiers of many physiological functions in human body (*90*).

Role of Other Bioactive Compounds

Different dietary phytonutrients including polyphenols are not essential for human body but a few of these molecules such as pro-vitamin A (β-carotene), vitamin K (phylloquinone) and vitamin E (tocopherols) are essential and play significant role in cellular metabolism (*96*). The physiological function of these phytonutrients is mostly governed by their antioxidative properties and they are able to counter oxidative stress by scavenging free radicals (*97*). There are many different forms of vitamin E presents in plants and all of them possess

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more or less equal antioxidant potential (*96*). Other than the antioxidant activity, tocopherols also can interact with enzymes, structural proteins, lipids and transcription factors (*96*). The association between serum concentrations of vitamin E and lipid peroxidation products in relation to cholesterol level and abdominal obesity has been observed in patients with metabolic syndrome (*24*). Tocopherol in combination with vitamin C counters free radicals and regulates vitamin E metabolism by recycling oxidized tocopherols (*98*). Many studies also reported that vitamin C has a specific role in oxidative-stress associated arterial hypertension and in regulating endothelial functions and vasodilation. Beckman et al. (*99*) observed that vitamin C administration was able to restore endothelium-dependent vasolidation in hyperglycemic patients.

The other important group of bioactive compounds are organosulfur compounds and found abundantly either in *Cruciferous* crops like radish, white mustard, black mustard, Brussels sprouts or in *Allium* sp. such as onions, garlic and leeks (*7*). Several studies have shown that garlic oil and raw garlic consumption could decrease total cholesterol, LDL cholesterol and triglyceride levels (*90*). Gore and Dalen (*100*) reported that consumption of 0.5 to 1.0 garlic per day lowers cholesterol levels approximately by 10%. Decrease in cholesterol and fatty acid synthesis and cholesterol absorption are mechanisms through which garlic favorably impact on human health. Other important bioactive compounds with protective properties include phytosterols, isothiocyanates and betaine. Betaine can act as a methyl donor in methionine cycle and inadequate dietary intake of betaine leads to disturbed hepatic methionine metabolism resulting in elevated plasma homocystein concentrations and increased serum lipid levels (*101*). Such metabolic alterations may contribute to coronary, cerebral, hepatic and vascular diseases. Natural bioactive compounds appear to have a role in regulation of serum glycemia and associated metabolic dysfunction and thus improvement of these compounds in plants can be an effective strategy in the management of chronic diseases including type 2 diabetes.

Emerging Dietary Strategies to Design Functional Foods for Disease Prevention

Nutritional and dietary therapy is potentially most effective tools to overcome the global burden of chronic diseases as health care costs are increasing rapidly. Development of functional foods by improving conventional foods with added health benefit properties is essential for future dietary applications. Phenolic phytochemicals plays crucial role in this novel approach and can be enhanced through different biochemical and biotechnological strategies (*102*). Novel tissue culture and bioprocessing technologies have been developed for consistent production of food grade phytochemical profiles (*103*). Phenolic compounds are secondary metabolites and derived through pentose phosphate, shikimate and phenylpropanoid pathways. The first committed step of pentose phosphate pathway (PPP) is carried out by glucose-6-phosphate dehydrogenase (G6PDH). The conversion to ribulose-5-phosphate in PPP also produces reducing equivalents (NADPH) for cellular anabolic reactions (*104*). Pentose phosphate pathway also

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generates erythrose-4-phosphate which along with phosphoenolpyruvate, from glycolysis, is channeled to the shikimate pathway to produce phenylalanine, which is directed through the phenylpropanoid pathway to produce phenolic compounds (*105*).

Shetty (*103*) proposed a model that proline-associated pentose phosphate pathway could stimulate both the shikimate and phenylpropanoid pathways, and therefore, the modulation of this pathway could lead to the stimulation of phenolic phytochemicals in plants (Figure 2). Using this model proline, proline precursors and proline analogues were effectively utilized to stimulate total phenolic content in plants (*106*, *107*). It was also proposed that demand for NADPH for proline synthesis during microbial interaction and proline analogue treatment may increase cellular NADP+/NADPH ratio, which should activate G6PDH. The proline analogue, azetidine-2-carboxylate (A2C), is an inhibitor of proline dehydrogenase and therefore tolerance to the analogue could stimulate proline synthesis, which drives the demand for NADPH (*108*). Another analogue, hydroxyproline is a competitive inhibitor of proline for incorporation of proteins. Either analogue at low level should deregulate proline synthesis from feedback inhibition (*105*). Therefore, deregulation of the pentose phosphate pathway may drive metabolic flux towards erythrose-4-phosphate for biosyntheis of shikimate and phenylpropanoid metabolites (*102*). At the same time, proline serves as a reducing equivalent, instead of NADH for ATP synthesis through oxidative in the mitochondria (*109*).

This model has been proposed for the mode of action of phenolic metabolites based on the correlation between stress stimulated phenolic biosynthesis and stimulation of antioxidant enzyme response pathways in plants (*103*). Within plant system model, acid, exogenous phenolic, proline analogues and precursor combinations and microbial elicitors were used to stimulate phenolic biosynthesis and key enzyme responses (*110*). Proline/G6PDH correlations during phenolic response were also associated with phenolic content, potential polymerization of phenolics by guiaicol peroxidase (GPX) and antioxidant activity based on free radical scavenging activity of phenolics and superoxide dismutase (*103*). So with the above mentioned rationale, it is possible to develop different dynamic strategies to harness the benefits of phytochemicals for the development of functional foods and nutraceuticals to counter chronic diseases like cardiovascular disease and diabetes.

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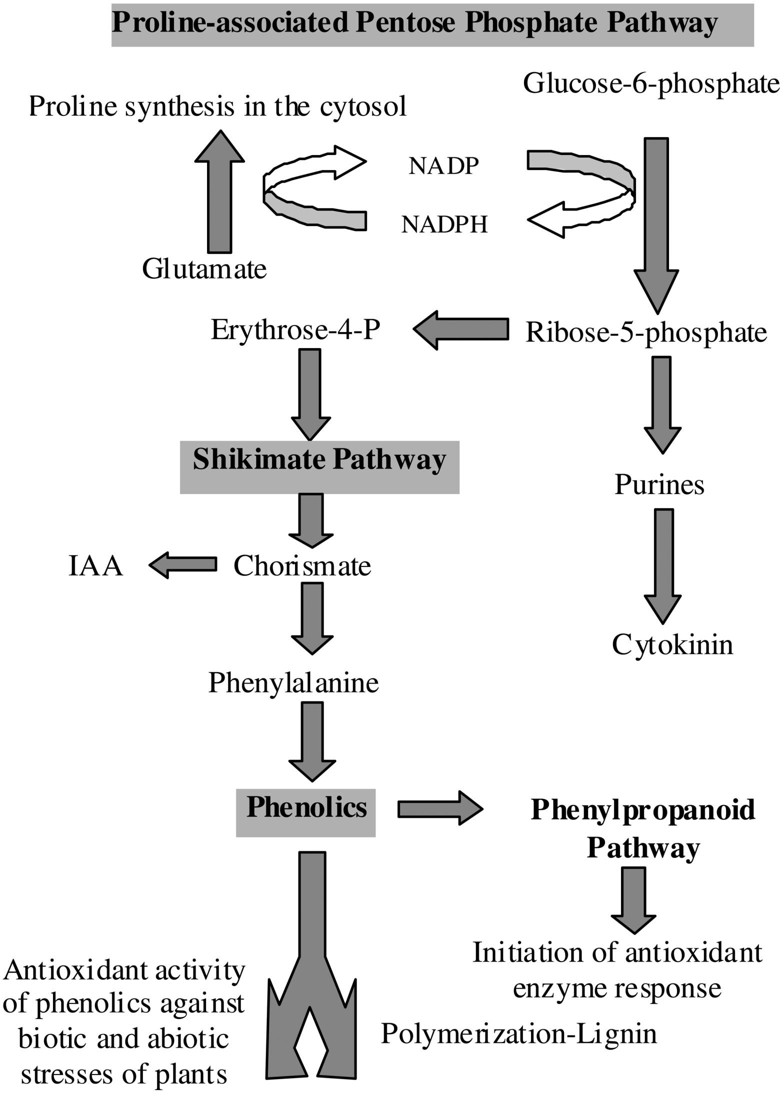
Proline-associated Pentose Phosphate Pathway
Proline synthesis in the cytosol
Glucose-6-phosphate
NADP
NADPH
Glutamate
Erythrose-4-P
Ribose-5-phosphate
Shikimate Pathway
Purines
IAA
Chorismate
Cytokinin
Phenylalanine
Phenolics
Phenylpropanoid Pathway
Initiation of antioxidant enzyme response
Antioxidant activity of phenolics against biotic and abiotic stresses of plants
Polymerization-Lignin

Figure 2. Phenolic biosynthesis in plants and its role in plant's biotic and abiotic stress.

Cereal, Grains, and Legumes

The primary polyphenols in cereal and legumes are flavonoids, phenolic acids and tannins. The bran and germ fractions derived from conventional milling process are rich source of biologically active compounds found in grain (*111*). Major nutrients in grain are vitamin B (thianin, niacin, riboflavin and pantothenic acid), minerals (calcium, magnesium, potassium, phosphorus, sodium and iron),

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basic amino acids (arginine and lysine) and elevated tocol levels in lipids (*111*). If all components of the kernel such as bran, germ and endosperm are present in natural proportion then it is considered as whole grains. Many studies reported a substantial inverse association between whole grain consumption and risk of chronic diseases including type 2 diabetes. Munter et al. (*112*) observed that high intake of whole grain particularly bran intake significantly reduced risk of type 2 diabetes in over 150,000 women. They also reported that increment of two-serving-per-day in whole grain was associated with a 21% decrease in risk of type 2 diabetes. Whole grains are known to slow digestion and absorption of carbohydrates and thus regulate postprandial blood glucose and insulin response. A Glycemic index (GI) of whole grain varies between 36 to 81, with barley and oats having the lowest value (*113*). Lower blood glucose levels and decreases insulin secretion have been observed in diabetic patients when consuming a low GI diet (=67). Heaton et al. (*114*) observed an increased plasma insulin response with whole grains. Juntunen et al. (*115*) reported that form of food and botanical structure are major determinant of grain for postprandial insulin response than amount of fiber or type of cereal in the food. The influence of whole grain on insulin response also governed through the beneficial effects on satiety and body weight. McKeown et al. (*116*) observed inverse association of whole-grain intake with body mass index and fasting insulin in Framingham offspring study. Sun et al. (*117*) reported that substitution of whole grains including brown rice for white rice lowered the risk of type 2 diabetes. Consumption of small grain like finger millet based diet resulted in significantly lower plasma glucose levels in six type 2 diabetes patients (*118*). The reduction of risk for type 2 diabetes may involves the synergistic effect of several whole grain components such as phytochemicals, vitamin E, magnesium and others (*119*).

Other than cereal, legumes are also an important part of our diet and have gained a prominent role to reduction in risk of chronic diseases. Legumes are rich source of protein, phytochemicals and minerals. High protein diet helps to improve insulin response and legumes are most safe source of dietary protein (*120*). Villegas et al. (*121*) reported that adherence to vegetables along with legumes such as soybean and peanut could reduce the risk of type 2 diabetes by 40% in a large Chinese population. Tang et al. (*122*) found some major antidiabetic components (soyasaponin and stigmasterol) in legumes (black soybean, white kidney bean and peanut) and their beneficial effects on the risk of type 2 diabetes. Soybean is most extensively studied legume for dietary and therapeutic applications. The major dietary polyphenols of soy responsible for pancreatic function and insulin secretion are isoflavonoids, genistein and daidzein (*86*). All of these phytochemicals preserved insulin production by the β-cells and contribute to improved insulin sensitivity in peripheral tissues and thus preventing pancreatic exhaustion (*86*). Fermentation with glucosidase-excreting fungi, natural elicitors and other biotechnological tools could improve phenolic phytochemical production and utilization of legumes as functional foods to counter chronic diseases (*123*).

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Fruits and Vegetables

High intake of fruits and vegetables has been associated with a reduced incidence of cardiovascular disease, diabetes and cancer. Daily consumption of fruits and vegetables in an adequate quantity (400-500 g/day) is recommended to reduce the risk of cardiovascular disease, stroke and high blood pressure (*7*). Research into neutraceuticals and functional food has highlighted the healthy compounds found in fruits and vegetables. For optimum health it is advised to consume five portions of fruit and vegetables (each comprising at least 80g) on a daily basis (*124*). Fruits and vegetables are rich in dietary fiber, folate, potassium, carotenoids and other phytochemicals. The high intake of fruits and vegetables lead to a reduced intake of saturated fat and cholesterol and thus prime candidates for protecting against diabetes (*125*). Fruits and vegetables are generally low energy dense foods, and if substitute for high energy dense, fatty and sugary foods, it can prevent obesity related chronic diseases like diabetes. Fruits and vegetables are rich source of polyphenols and antioxidant capacity of fruits and vegetables dictate their beneficial role against chronic diseases. The major contribution of fruit and vegetable intake to preventing diabetes is mediated through reductions in overweight and obesity (*126*). Many epidemiological studies have shown an inverse relationship between consumption of fruits and vegetables with incidence of cardiovascular disease and diabetes (*127*).

Several studies have been conducted in last two decades to assess the role of fruits and vegetables on the incidence of type 2 diabetes. Increasing the amount of leafy vegetables could help to reduce the risk of type 2 diabetes (*126*). Consumption of five portions a day of fruits and vegetables reduced the risk of type 2 diabetes by 40% in women (*128*). In another study with Finish men and women showed a higher intake of fruit and berries diminished the risk of diabetes by 30% (*129*). Villegas et al. (*121*) observed an inverse relationship between vegetable intake and incidence of type 2 diabetes in Chinese women. Liu et al. (*130*) reported that high intake of green leafy or dark yellow vegetables were associated with reduced risk of type 2 diabetes among overweight women. Green leafy vegetables contain high concentrations of β carotene, vitamin C, polyphenols which are known for their antioxidant properties. They are also good source of magnesium and α linolenic acid, which is an omega 3 polyunsaturated fatty acids (*131*, *132*).

Other than epidemiological and cohort studies, some studies also shown association between individual vegetables and risk of type 2 diabetes. McCue et al. (*76*) investigated the potential of phenolic-optimized aqueous extracts of selected foods of American and Asian diet for antidiabetic potential. They found that common vegetables and spices contained significant antidiabetic (α-amylase and α-glucosidase) activity in *in vitro* assays. Vegetables from *Solanaceae* family including pepper, eggplant and tomato are rich source of phenolic phytochemicals with high antioxidant activity and beneficial on human health. Kwon et al. (*133*) observed that several pepper (green, red, orange, yellow, Cubanelle, red sweet, yellow sweet, long hot and Jalapeño) extracts had high α-glucosidase inhibitory activity and low α-amylase inhibitory activity . They also found that among all these pepper extracts, yellow, Cubanelle and red pepper had highest angiotensin

I-converting enzyme (ACE) inhibitory activity. Eggplant is also recommended as an important dietary source for management of type 2 diabetes (*77*). High α-glucosidase inhibitory activity and moderate to high ACE inhibitory activity was observed in phenolic-enriched extracts of eggplant. Similarly, Saleem et al. (*134*) found that some selected line of sub-tropical cultivars of Chilean potatoes had anti-hyperglycemia (moderate α-glucosidase and no α -amylase inhibitory activity) and anti-hypertensive (moderate to high ACE inhibitory activity) potential. These findings could help to develop vegetable-based whole foods for dietary management of type 2 diabetes and hypertension.

Like vegetables, fruits are also rich source of dietary polyphenols and associated with reduction in type 2 diabetes incidence and coronary mortality. Among polyphenols, anthocyanins are responsible for variety of bright colors in fruits and present in various foods and beverages. These compounds are well known free radical scavengers and associated with a reduced risk of degenerative diseases including diabetes. Jayaprakasham et al. (*135*) found that several anthocyanin compounds could stimulate insulin secretion from rodent pancreatic β-cells. Cherries are rich source of bioactive anthocyanins and thus possess higher lipid peroxidation and cyclooxygenase enzyme inhibitory activity (*136*).

Seymour et al. (*137*) observed that administration of cherry-enriched diet for 90 days significantly reduce fasting blood glucose, hyperlipidemia, hyperinsulinemia and fatty liver in rats. High phenolic content, high total antioxidant activity and high α-glucosidase inhibitory activity was observed in different sweet and tart cherry cultivars (*138*). Tart and sweet cherry could reduce several phenotypic risk factors that are associated with risk for metabolic syndrome and type 2 diabetes.

Berries are rich source of polyphenols, such as anthocyanins, flavonols, phenolic acids, ellagitannins and procanthocyanidines. Berries are excellent fruit choice for diabetes because of their low caloric content and presumed favorable glycemic response. Wilson et al. (*139*) found that Sweetened dried cranberries with low sugar contain fewer calories and could be used to improve fruit consumption by person with type 2 diabetes. Polyphenol-rich extract of blueberries, blackcurrants, strawberries and raspberries have been shown to inhibit α-glucosidase activity *in vitro* (*72–74*). Cheplick et al. (*72*) reported high α-glucosidase inhibitory activity for yellow raspberry cultivars, suggesting that the α-glucosidase may be influenced more by specific anthocyanins rather than the actual amount of the overall total plant phenolics. Pinto et al. (*74*) studied the potential effects on the in vitro inhibition of α-amylase and α-glucosidase enzymes from different Brazilian strawberry cultivars. They found that strawberries had high α-glucosidase and low α-amylase inhibitory activities, suggesting these fruits as good sources for potential management of hyperglycemia linked to type 2 diabetes as a part of an overall diet.

Apple one of the main sources of flavonoids in western diet provides approximately 22% of the total phenols consumed per capita in the United States (*140*). An increased intake of apple has been correlated with decreased risk of cardiovascular disease and diabetes (*140*). Huber and Rupasinghe (*141*) reported that apple skin extracts particularly from crab apple cultivars could be effective inhibitors of oxidation of polyunsaturated fatty acids and thus considered as a

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good source of natural food antioxidants. Adyanthaya et al. (*142*) observed a positive correlation between phenolic content and high α-glucosidase inhibitory activity in different apple cultivars and it also enhanced post-harvest preservation. Phenolic-enriched apples may modulate postprandial glucose levels and thus reduce the risk of type 2 diabetes developments. Barbosa et al. (*143*) also found that phenolic enriched apple cultivars had high α-glucosidase inhibitory activity. The main phenolic compounds found in peel extracts were quercetin derivatives, protocatecuic acid and chlorogenic acids, where pulp extracts had quercetin derivatives, chlorogenic acid and *p*-coumaric acid. High phenolic content and α-glucosidase activity was also observed in peel and pulp extracts of different pear cultivars (*144*). Phenolics-enriched fruits like apple, pear, cherry, and berries not only control postprandial hyperglycemia but also maintain cellular redox balance to prevent long term diabetic complications. Pinto et al. (*145*) reported high α-glucosidase and ACE inhibitory activity of some Peruvian fruits. Modulation during pre- and post-harvest stages through agronomic, horticultural, biotechnological and bioprocessing tools could improve bioactive compounds in these fruits and could be utilized in effective therapeutic strategies in type 2 diabetes and hypertension management.

Medicinal Plants and Herbs

Herbs are rich in phenolic phytochemicals and have been used as a source of food and medicine since ancient times. High antioxidant activity and other therapeutic properties of medicinal herbs offer cost effective strategies to control postprandial hyperglycemia with minimum side effects (*146*). Acarbose and miglitol currently used to control blood glucose levels and work by reversibly inhibiting digestive α-amylase and α-glucosidase enzymes. Abdominal distention, flatulenece, meteorism and diarrhea are major drawbacks in use of acarbose as drug (*147*). Medicinal plant like *Salacia reticulata* used traditionally in *Ayurvedic* medicine has many antidiabetic properties. Oral administration of *S reticulata* extracts significantly reduced blood glucose levels after a carbohydrate meal in rats (*148*). *Salacia* extracts have also shown to be an effective treatment for type 2 diabetes in human patients (*149*). This plant extract is also effective in reducing body weight gain and thus reduce the risk of obesity associated complications (*150*). Sim et al. (*151*) observed that de-O-sulfonated kotalanol from the salacinol class of compounds in *Salacia* was potentially stronger α-glucosidase inhibitor compared to miglitol and acarbose.

Plants from *Lamiaceae* family (Mint family) including sage, rosemary, lavenders, oregano have many medicinal properties and have long been used in food preservation, culinary flavors and aromas (*146*). These herbs are also rich source of phenolic phytochemicals and have high antioxidant activity. Kwon et al. (*146*) observed high α-glucosidase inhibitory activity in water extracts of oregano, chocolate mint, lemon balm, sage and clonal line of rosemary and this result is positively correlated with its phenolic content. Major phenolics detected in this study were catechin, caffeic acid, rosmarinic acid, resveratrol, catechol, protocatechuic acid and quercetin (*146*). They also observed high ACE inhibitory activity in the water extracts of rosemary, lemon balm and oregano (*146*). Saleem

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et al. found high α -glucosidase activity in aqueous extracts of marjoram and sage (*152*).

Another important plant family with high medicinal values is *Apiaceae* and major plants in this family are carrot, coriander, caraway, anise, fennel, dill, and ajowan. Caraway is probably involved in lipid metabolism and thus has beneficial effect for treatment of diabetes and cardiovascular disease (*153*). Saleem et al. (*154*) observed moderate to high α-glucosidase inhibition and high antioxidant activity in different *Apiaceae* family seed extracts. This *in vitro* study has shown that *Apiaceae* family seed extracts could be used as food condiments and could have antidiabetic potential. Kwon et al. (*155*) observed high α-glucosidase inhibitory activity in tyrosol, a major phenolic component of *Rhodiola* plant. *Rhodiola* extract have also shown ACE inhibitory activity and could be used as a dietary supplement in postprandial hyperglycemia and hypertension management. Apostolidis et al. (*156*) also observed high α-amylase, α-glucosidase and ACE inhibitory activity in *Rhodiola* extracts alone or in combination with cranberry and oregano extracts. Leaf extract of *Nerium indicum* and *Gingko biloba* showed significant reduction of the postprandial rise in blood glucose levels after oral administration in sucrose-loaded rats (*157*, *158*). There are many geographical areas (Himalaya, Andes, Amazon, Western ghat of India, East Asia) in the world with rich source of plant diversity and many of these species are still unexplored in terms of their medicinal applications. It is very important to identify and examine the bioactive compounds of such species for future management of chronic diseases through application in diet and natural medicine.

Fermented Foods

Fermentation is defined as a process of conversion of complex organic compounds into simpler forms in the absence or near absence of oxygen. It is one of the oldest, most efficient, and low cost processes which can be broadly classified as solid state fermentation and liquid fermentation (*159*).

Health benefits of fermented foods has been known to mankind since thousands of years however recent studies on the health benefits of fermented foods ranging from its anti-mutagenic properties to its cholesterol lowering effects have renewed the interest in exploring and developing novel fermentation based strategies for additional health benefits (*160*). Phenolic phytochemicals with their antioxidant ability and carbohydrate uptake enzyme (α-glucosidase and α-amylase) inhibition potential offer an attractive strategy to enhance foods with these compounds as a possible way to prevent hyperglycemia and related oxidative diseases. Fermentation based bioprocessing as a novel tool to design functional foods to prevent and combat type 2 diabetes has been described.

a. Solid State Bioprocessing

Miso, Natto, Tempeh, Soy-sauce are some of the examples of traditional foods that make use of the growth of aerobic fungi on complex organic substrates as a part of a multi-step process to preserve and enhance the functionality of its products. Phenolic enrichment could take place by enzymes produced by the fungus in an attempt to utilize the substrate for growth with or without contribution from the fungus itself (*161*). Carbohydrate cleaving enzymes such as β-glucosidase, α-amylase, α-glucosidase, β-glucuronidase have been attributed to phenolic antioxidant mobilization during solid state bioconversion of legumes and cereals (*162–165*). Insoluble phenolics are solubilized by β-glucuronidase whereas β-glucosidase and α-glucosidase are involved in mobilization of carbohydrate conjugated phenolics (*162*). Increased phenolic content in defatted soybean powders by *Lentinus edodes* during solid state bioprocessing has been correlated with increased β-glucosidase, and α-amylase activity which suggests β-glucosidase along with lignin degrading enzymes breakdown polymeric layers to increase the accessibility of starch for the fungus via amylase activity (*163*). During solid state bioprocessing of soybean carbohydrate cleaving enzymes catalyze the formation of isoflavonoid aglycones which have been reported to have more potential than their corresponding glycosides for regulating glucose metabolism (*166*). Douchi, a microbial processed soybean product has been shown to have anti-hyperglycemia potential in mice due to the presence of genistein a potent α-glucosidase inhibitor (*167–170*). Increased pancreatic α-amylase inhibition in fungal enriched, Roquefort cheese was attributed to specific phenolics or secondary metabolites produced by *Penicillium* species (*171*). Solid state bioprocessing concept has been further extended for production of phenolic antioxidants from fruit substrates such as cranberry pomace and pineapple waste (*161*, *172*).

b. Liquid State (Submerged) Fermentation

Lactic acid bacteria and yeast or mixed (Kefir) cultures have been studied for phenolic antioxidant mobilization in milk, soymilk and fruit juices. Apostolidis et al., (*173*) describe an increase in *in vitro* antioxidant activity in milk and soymilk fermented with *Lactobacillus bulgaricus* with an increase in glucose uptake enzyme (α-glucosidase and α-amylase) inhibitions. In a similar study with Kefir culture mediated fermentation of soymilk supplemented with *Rhodiola* extracts an increase in α-glucosidase inhibition was observed which correlated to increased tyrosol and reduced salidroside contents (*174*). Antioxidant activity and *in vitro* α-glucosidase inhibition potential has been shown to correlate with the phenolic content in blueberry yogurt (*171*). A small, water soluble compound in Kefram-Kefir was determined to be the active agent in augmenting glucose uptake in L6 myotubules both in the presence and absence of insulin (*175*). Similarly in rats fed on a high fructose diet, glucose intolerance was delayed by one-two weeks when their diet was supplemented with skim milk and yogurt as compared to impairment of glucose tolerance at the third week in control group (*176*). Phenolic antioxidant mobilization during yogurt production from soymilk using Kefir cultures strongly correlated with polymeric phenolic degrading enzymes, peroxidase and laccase (*177*). During fermentation of soymilk with lactic acid bacteria and bifidobacteria a significant increase in alglycone isoflavone content along with an increase in antioxidant activities as

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compared to unfermented milk was observed (*178*, *179*). During fermentation of berry juice with a novel bacterium *Serratia vaccinii* an increase in antioxidant activity which not only associated with an increase in total phenolics but also with a change in phenolic profile. The increase in antioxidant activity was attributed to the enzymatic activity of the bacterium causing deglycosyaltion of glycosylated phenolic compounds whereas increase in phenolics was attributed to degradation of tannins and/or biosynthesis of new phenolic compound by the bacterium (*180*). Blueberry juice fermented with *S. vaccinii* increased glucose uptake in myotubules and adipocytes whereas nonfermented juice had no effect, suggesting fermentation with *S. vaccinii* confers antidiabteic properties to the juice (*181*). Spectrofluorometric determination of antioxidant ability of fermented papaya extract indicated it is a potent antioxidant and might alleviate symptoms associated with oxidative stress in severe forms of thalassemia (*182*).

We recently extended the concepts of lactic acid bacteria based milk fermentations and bacteria based berry juice fermentation to *Rosaceae* family (Apple, Pear and Cherry) juice fermentation with *Lactobacillus acidophilus* to determine phenolic mobilization, antioxidant capacity and anti-hyperglycemia potential (*177*). Fresh press juice (100%) was adjusted to a pH of 6 and then fermented with *Lactobacillus acidophilus* for 72 h and the changes in total soluble phenolic, antioxidant potential along with inhibition of key starch breakdown and uptake enzymes were determined every 24h. Analysis was carried out at every time point by adjusting the pH and not adjusting the pH (fermented acidic pH). Total phenolics, antioxidant activity decreased over a period of 72h with a temporary spike at 48h. Total phenolic present in the fermenting substrate is a result of phenolic mobilization linked to a flux between the formation/degradation of polymeric phenolics and the liberation of free phenolics. The decrease could also be because of degradation of phenolic compounds as a possible detoxification mechanism for lactic acid bacteria or because of formation of large polymeric phenolics from simple phenolics during fermentation. Decrease in antioxidant capacity may be due to changes in phenolic structures caused by the bacteria or their enzymes during fermentation. Alpha-Glucosidase inhibition decreased overall for pH adjusted samples whereas it increased for fermented acidic samples in case of apple. In case of pear an increase in α-glucosidase inhibition was found with fermented acidic samples whereas for pH adjusted samples inhibition decreased or remained constant. Mixed results were obtained with cherry depending on the pH treatment before and after fermentation. Fruit juice fermentation by beneficial bacteria and probiotics not only has the potential to alleviate anti-hyperglycemia and related diseases but also have a dual function of promoting better gut health and innate immunity. More research is required on the effects of different cultivar and strain based fermentations along with studies on the translations of these effects *in vivo* to better comprehend the impact of fruit juice fermentations on oxidative stress-linked diseases and related complications.

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Future Directions

All of the above rationales suggest that diet and plant-based foods play a crucial role in managing and combating chronic diseases and better dietary strategies with value added components may provide efficient and effective management options. For designing functional foods and nutraceuticals, the major challenge is obtaining consistent and optimized content of bioactive compounds in the right whole food system. Agronomic, physiological and genetic manipulations can enhance and optimize the content of phytochemicals by stimulating preferential biosynthesis through metabolic pathways specific to these desired bioactive compounds. It is important to know the role of environmental factors on the deposition of phytochemicals as plants produce these compounds for their own defensive mechanisms. Biotic and abiotic stresses induce the biosynthesis of phytochemicals including polyphenols and manipulation of such environmental factors (low temperature, heat, UV-B radiation, salinity, water stress and pathogens) can stimulate their production at right maturity and post-harvest stages. Many studies have been reported that under controlled environment alteration of one of such stress components could enhance bioactive phenolic phytochemical production concurrent with induction of antioxidants enzyme response in plants. Such whole plant responses coupled to the antioxidant enzyme response could help optimize desired bioactive profiles as chronic disease conditions are closely associated with oxidation malfunctions.

Phytochemical stimulation through natural elicitors is another means to enhance the production of bioactive compounds. Natural elicitors mimic the parallel environmental challenges and thus trigger physiological and morphological responses, which results in the accumulation of phytochemicals. Abiotic and biotic elicitors such as metal ions, inorganic compounds, fungi, bacteria, plant cell wall component and natural antioxidant stimulator can be used in different growth and developmental stages of plant to optimize the production of bioactive compounds (*183*). Accumulation and induction of potential health beneficial compounds such as glucosinolates, indole alkaloids, tarpenoids, flavonoids and phenylpropanoids have been observed in plant cell culture with natural elicitor treatment (salicylic and jasmonic acid) (*7*). The other agricultural practices including time of harvest, type and rate of fertilization, maturity of plant, postharvest conditions also influence the concentration of bioactive compounds in plants (*184*). The maintenance of phytochemicals in fruits, vegetables and medicinal herbs after harvest is also another major challenge. Different strategies including alterations in storage environment, physical and chemical treatments and biotechnological tools are emerging for the improvement of phytochemical content in plant products during postharvest stages. Still there is a strong need for future research in this area as deterioration in the quality parameters during this stages is often a major limitation for marketing and utilizations and there are common responses between post-harvest preservation and associated phytochemicals with health benefits.

Genetic control also plays crucial role in the production of functional metabolites and thus conventional and biotechnological breeding strategies can be an effective tool for manipulation and augmentation of functional phytochemicals..

Screening of different species and cultivars with enhanced levels of specific phytochemicals is the primary step in the conventional breeding approach (*7*). Many different cultivars and clonal lines with enhanced phytochemical content have been developed using conventional breeding techniques (*185*). Genetic engineering including transcriptional factors of secondary metabolic pathway by targeting activity of specific enzyme are believed to be an effective strategy for the development of the phenolic-enriched plant foods (*186*). Although there has been some success in the metabolic engineering related to phytochemical production but over-expression has sometimes resulted in the production of unexpected end products (*7*). It is important to consider potential safety and environmental issues at the time of choosing and using such technologies. Future research in genetic engineering of bioactive compounds should emphasize the importance of diet-health-environment paradigm.

There is also growing concerns about the synergistic effect of different bioactive compounds and effect of different combinations of food components on human health. When we consume diet several endogenous and exogenous factors dictate the beneficial role of bioactive compounds and the mechanism of such regulation is complex. To harness maximum benefit of diet for disease prevention it is necessary to understand the interactions and regulations of food, microbes and gut enzymes. Many researches have been conducted with single bioactive compound and by targeting specific enzyme activity through *in vitro* studies. There are significant limitations in such studies and there is need to improve through animal and human models for further justification and rationale for whole food designs for functional benefits based on specific health target..

Socio-cultural, economic and educational factors are also critical for the development of different disease prevention strategies. Community development and localized food security programs with major focus in the role of diet, fresh foods and dietary components on human health could be very effective. Education about food systems, nutrition and health awareness program among populations with different age, socio-cultural and economic background is also required for sustainable management and prevention of chronic diseases. A proper regulation in marketing of food products and revelation of nutritional facts in packaged foods is also necessary to avoid any discrepancy and false assumptions on health foods. To combat the global burden of noncommunicable chronic diseases it is important to integrate all above mentioned factors, which will result into the development of a healthy dietary system with good compliment of health-linked and bioactive-enriched plant-based foods as a part of overall balanced diet.

Conclusions

Type 2 diabetes and other noncommunicable obesity-linked chronic diseases like cardiovascular disease and cancer are predominant threat for the present and future global populations. The understanding of the physiological and biochemical mechanisms and pathophysilogy of these diseases have revealed that changes of dietary pattern through the natural and anthropogenic evolution resulted into this rapid emergence of diet-linked chronic diseases. Diet rich in

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plant based phytochemicals can be a major tool for the prevention of chronic diseases including type 2 diabetes. These bioactive compounds play a significant role in glucose metabolism and manipulation of these phytochemicals through different biochemical and biotechnological strategies can develop functional foods with high therapeutic benefits to combat these preventable chronic diseases. . Design of such healthy diet along with other environmental and lifestyle improvement can help to maintain healthy cellular homeostasis and can counter oxidative stress induced metabolic disorders in the human body. Nutritional and therapeutic approaches comprising of plant-based healthy diet can be a safe, cost effective and sustainable option for effective management of type 2 diabetes and its oxidative and vascular complications. Scientific advancement in plant-based functional food research and integration of socio-economic and policy strategies will lead to effective integrated strategies to prevent and manage overall global burden of these chronic diseases.

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Chapter 21

Urinary Pharmacokinetics of Queen Garnet Plum Anthocyanins in Healthy Human Subjects

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A new variety of *Prunus salicina*, Queen Garnet plum (QGP), was developed as a high anthocyanin, high antioxidant plum, in a Queensland, Australia, Government breeding program. In this manuscript, we are presenting for the first time data about the urinary pharmacokinetics of QGP anthocyanins and derived metabolites in healthy humans. Following consumption of 400 mL QGP juice (QGPJ; 2.49 mmol anthocyanins) by two male subjects, QGP anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside) were excreted mainly as methylated glycosides, glucuronides, and sulfoconjugated metabolites in urine. The cumulative excretion of anthocyanins could be fitted to a one-compartment pharmacokinetic model with instantaneous, parallel excretion of anthocyanin metabolites. The usefulness of this non-linear modeling statistical

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technique for characterizing the urine excretion-time profiles and estimating relevant PK parameters is demonstrated. Results from this pilot study indicate that methylation and glucuronidation are significant metabolic routes when cyanidin-based anthocyanins are consumed in QGPJ. Future studies investigating the benefits of the consumption of anthocyanin-rich Queen Garnet plums and/or derived products should therefore focus on identifying anthocyanin metabolites and include their putative colonic degradation products. This will assist in evaluating the biological relevance of these compounds to health and disease prevention.

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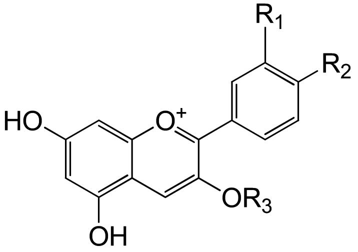
Introduction

Anthocyanins, a polyphenol subclass, are one of the most abundant phenolic compounds in nature and are responsible for the red, purple, and blue colours of many fruits and vegetables, including plums. Approximately 640 individual anthocyanins have been identified to date (*1*). Anthocyanins also provide the food industry with natural alternatives for some synthetic food colourants to address the increasing public concern about synthetic food dyes (*1–3*). The six anthocyanidins (aglycons) commonly found in plants are classified according to the number and position of hydroxyl and methoxyl groups on the flavan nucleus and are named pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin (*4*). Numerous studies, mostly *in vitro,* and some animal experiments, have demonstrated a broad range of biological properties for anthocyanins, including antioxidant, anti-inflammatory, antimicrobial, and anti-carcinogenic activities (*4*). Their daily intake has been estimated at between 12.5 mg in the United States (*5*) and 83 mg in Finland (*6*) which is considerably higher than the intake estimated for many other polyphenols. The evaluation of the bioavailability and pharmacokinetics (PK) of anthocyanins has recently been gaining significant interest as consumers seek foods with potential disease prevention benefits in addition to their nutritional value ("functional food") (*1*, *7*). However, detailed information on the absorption and metabolism of anthocyanins from raw and processed food is still limited. Several studies have found that anthocyanins are absorbed as intact glycosides and appear in blood and urine, whereas some other studies have found anthocyanins to be present mainly as metabolites (*4*, *8*, *9*).

Studies on anthocyanin bioavailability, as measured by absorption and excretion of intact anthocyanins and their main metabolites, appear to be very low and usually much less than 1% (*10*). The identification of the metabolic fate of these plant pigments, including their biotransformation by intestinal bacteria, as well as establishing their PK, is a basic requirement to evaluate their biological relevance to health and disease prevention. Furthermore, there is now emerging evidence that *in vivo* metabolites of dietary anthocyanins and other polyphenols, which appear in the circulatory system in a nanomolar to low micromolar range, can exert modulatory effects in cells through several effects on the intracellular signalling cascades (*11*).

A new variety of the Japanese plum *Prunus salicina* Lindl., named Queen Garnet plum (QGP), was developed within a Queensland, Australia, Government breeding program (*12*). The strategy of this breeding program was to produce a high yielding plum with an outstanding anthocyanin content and antioxidant capacity. The anthocyanins present in QGP and QGP juice (QGPJ) were identified previously as cyanidin-3-glucoside and cyanidin-3-rutinoside (Figure 1) (*13*).



Anthocyanin	R1	R2	R3
cyanidin 3-glucoside	OH	OH	Glucose
cyanidin 3-rutinoside	OH	OH	Rutinose

Figure 1. Chemical structures of anthocyanins identified in QGPJ; Rutinose is 6-O-α-L-rhamnosyl-D-glucose.

In contrast to the development of synthetic drugs, there is no regulatory need to study the PK of food ingredients. Nonetheless, most pharmacokinetic principles of synthetic drugs are considered to be applicable to phenolic compounds from raw or processed food (*14*). Metabolism of xenobiotics including anthocyanins has been regarded as one of the most important and complex processes in the body, leading to the excretion of those substances. To optimize the use of anthocyanins from raw or processed food (e.g. dosage, regimen and administration route), knowledge on their biological fate including the disposition pathways and kinetics in the human body is needed to establish potential dose-effect and dose-concentration relationships. At a later stage this knowledge would allow for conducting proper therapeutic monitoring for anthocyanins. Pharmacokinetic studies of anthocyanins from food also provide information of potential food-drug interactions.

Although the typical pharmacokinetic analysis is based on data from blood samples, modeling of urine samples is seen as an important area (*15*). Urine collection is less invasive than blood sampling and does not require subject presence at the clinic site at the time of sample collection as voids can be collected and retained under appropriate storage conditions for later delivery to the clinic site or laboratories. The objective of the present study was (i) to

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investigate the absorption, metabolism, and excretion of QGP anthocyanins and derived metabolites in two healthy human subjects, and (ii) to characterize the concentration profiles of anthocyanins and metabolites in urine as a basis for improving and simplifying bioavailability studies, as urine collection can be made at an optimal time to capture the exposure to anthocyanins.

Material and Methods

Chemicals

Unless otherwise stated, all chemicals were purchased from Merck (Kilsyth, VIC, Australia) or Sigma-Aldrich (Castle Hill, NSW, Australia), and were of analytical or HPLC grade. De-ionized water was used throughout. Cyanidin-3-glucoside and cyanidin-3-rutinoside were purchased from ChromaDex (Irvine, California, U.S.A.).

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QGPJ Preparation

QGP which had been stored at -20°C was thawed and manually halved and de-pitted. QGP was then placed into a bowl chopper for 30 s and heated in a steam vat to 50°C before enzyme treatment with a commercial pectinase (PectinexMash, 200ppm, Novozymes, Sydney, Australia). The QGP puree was incubated at 50°C for 1 h before being juiced in a screw press (Brown International Corporation, Model 3600). Collected QGPJ was then pasteurized (by heating to 80°C and holding for 5 min) and stored at -10°C prior to ingestion. Microbial analysis (plate count) was undertaken on QGPJ to ensure juice was fit for human consumption.

Pilot Study with Healthy Human Subjects

Ethical permission was obtained from the CSIRO Human Experimentation Ethics Committee (code 08/22). Two healthy, non-smoking male volunteers were recruited; subject 1: 26 years, body weight 80 kg, body height 1.88 m, subject 2: 41 years, 74 kg, 1.70 m. Participants adhered to their usual diet, but abstained from food and beverages rich in polyphenols and ascorbic acid from 24 h prior to treatment. Alcohol and medication, including over the counter drugs, were refrained from consumption during the course of the pilot study. Both subjects had the following experimental treatments by which each subject served as his own control: 400 mL of QGPJ (containing 2.49 mmol of anthocyanins expressed as cyanidin-3-glucoside equivalents) or 400 mL of water as an anthocyanin-free control beverage separated by a one week wash-out phase. At 8.00 a.m. after an overnight fast, volunteers took 400 mL of one of the two beverages, respectively, together with white bread rolls. During the experimental periods, only the consumption of water and of three further standardised meals (white bread rolls with cheese for lunch, afternoon snack, and dinner) was allowed. Urine samples were collected pre-dose and quantitatively in six intervals up to 24 h after dosing (0-2, 2-4, 4-6, 6-8, 8-10, and 10-24 h). Aliquots of 7 mL were acidified and stabilised with conc. formic acid (1:1.3) and stored frozen at -80°C until analysed.

Analysis of Anthocyanins

Anthocyanins in QGPJ

QGPJ was diluted 1:100 with HPLC mobile phase A (water/formic acid/acetonitrile, 87:10:3, v/v/v) and filtered through a 0.45-µm GHP Acrodisc Minispike filter (Pall Life Sciences, MI, U.S.A.) before injection into the HPLC. The juice was analysed in triplicate with the HPLC method and system described below.

Anthocyanins and Derived Metabolites in Urine

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Urinary anthocyanins and metabolites were extracted with a solid-phase extraction (SPE) cartridge (Sep-Pak C18; Waters, Milford, MA) according to Felgines *et al.* (*16*) with slight modifications. Acidified urine samples were thawed and maintained for 60 min at room temperature before SPE extraction to obtain the maximal yield of the coloured flavylium cations. The SPE cartridge was activated with 10 mL of methanol and equilibrated with 10 mL of 12 mM aqueous HCl before use. Subsequently, 9 mL of acidified urine was applied to the equilibrated cartridge. The cartridge was then washed with 10 mL of 12 mM aqueous HCl, and anthocyanins were eluted with 15 mL of 12 mM HCl in methanol. The methanolic extract was evaporated under nitrogen to a volume of 1 mL by use of a Rotavapor R-210 (Buchi, Flawil, Switzerland) at 35°C. Aliquots of 1 µL (for identification of anthocyanins and anthocyanin metabolites) and of 50 µL (for quantification) were used for HPLC.

Analysis of anthocyanins and anthocyanin metabolites was carried out by HPLC-PDA according to the method of Kammerer *et al.* (*17*) with slight modifications. The HPLC system consisted of a 600E multisolvent delivery system (pump), in line-degasser AF, 2996 photodiode array detector (PDA), 717 plus auto-injector and 600 system controller (Waters Corporation, Milford Massachusetts, U.S.A.) equipped with an Aqua Luna C18(2) (250 x 4.6 mm i.d.) reversed phase column with a particle size of 5 µm (Phenomenex, Lane Cove, NSW, Australia) protected by a Phenomenex 4.0 x 3.0 mm i.d. C18 ODS guard column. The column was operated at a temperature of 25°C. The mobile phase consisted of water/formic acid/acetonitrile (87:10:3, v/v/v; eluent A) and of acetonitrile/water/formic acid (50:40:10, v/v/v; eluent B). The gradient program was as follows: 10% B to 25% B (10 min), 25% B to 31% B (5 min), 31% B to 40% B (5 min), 40% B to 50% B (10 min), 50% B to 100% B (10 min), 100% B (5 min. isocratic), 100% to 10% B (1 min), and 10% B for 5 min before injecting the next sample. The injection volume was 50 µL and samples were filtered through a 0.45-µm GHP Acrodisc Minispike filter prior to injection. Detection was carried out at 520 nm at a flow rate of 0.8 mL/min. Anthocyanins and individual anthocyanin metabolites were quantified by comparison of their peak areas with an external calibration curve obtained using known concentrations of cyanidin-3-glucoside. The cumulative amount of compound excreted into urine within 0 to 24 hours ($A_{e(0\text{-}24)}$) was calculated according to Eq. (1).

$$A_{e(0\text{-}24)} = \Sigma(C \times V) \quad (1)$$

where C is the concentration in the collection interval and V the fractional urine volume. The fraction of orally administered compound excreted into urine within 24 hours (f_e/f) was calculated according to Eq. (2).

$$f_e/f\,(\%) = A_{e(0\text{-}24)}/Dose \times 100\% \quad (2)$$

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Results were expressed as micromoles (µmol) or nanomoles (nmol) of cyanidin-3-glucoside equivalents. It should be noted that the present study focused on only anthocyanins (unchanged glycosides) and anthocyanin metabolites having an intact flavylium skeleton and being thus detected at 520 nm.

LC-PDA-MS analysis was carried out on a Quantum triple stage quadrupole (TSQ) mass spectrometer (ThermoFinnigan, NSW, Australia) equipped with a quaternary solvent delivery system, a column oven, a photo-diode array detector and an autosampler. An aliquot (20 µL) of a methanolic solution (250 µg/mL) was chromatographed on a Luna C18(2) column (150 x 2.1 mm, 5 µm particle size), (Phenomenex, NSW, Australia) which was maintained at 25 °C. The mobile phase consisted of 1% formic acid in water (A) and 1% formic acid in acetonitrile (B) at the rate of 0.3 mL/min (no splitting). A linear gradient was used (10% B to 100% B over 40 min). Ions were generated using an electrospray source in the positive mode under conditions set following optimisation using solutions of cyanidin-3-glucoside. MS experiments in the full scan (parent and product-specific) and the selected reaction monitoring (SRM) mode were carried out to confirm the identity of components.

All analysis and determinations were performed in duplicate unless otherwise stated.

The non-linear modeling approach wherein estimated PK parameters derived from fitting an appropriate PK model was used to determine the PK of intact QGP anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside) and their metabolites (sum of cyanidin monoglucuronide, cyanidin monosulfate, peonidin monoglucuronide, peonidin-3-glucoside, and peonidin-3-rutinoside).

The cumulative urine excretion of intact QGP anthocyanins and their metabolites was evaluated by using the WinNonlin Professional 5.2.1 program (Pharsight Corporation, Mountain View, CA, U.S.A.). A one-compartment model with parallel linear elimination kinetics and first-order input (Figure 2) was fitted to the experimental cumulative excreted amount-time data for each subject.

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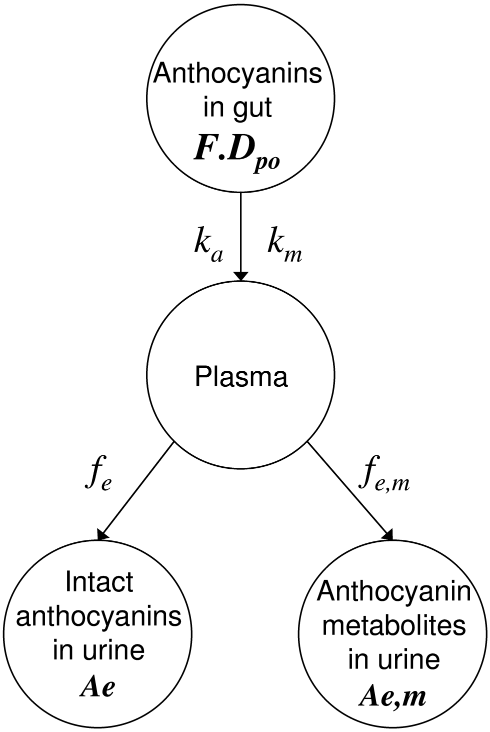
Anthocyanins in gut
F.D_po
k_a
k_m
Plasma
f_e
f_e,m
Intact anthocyanins in urine
Ae
Anthocyanin metabolites in urine
Ae,m

Figure 2. Schematic illustration of the one-compartment model with parallel elimination of intact anthocyanins and their metabolites into urine. A fraction of the anthocyanin is converted to a metabolite, which is then instantaneously excreted via the kidneys. Further explanation is given in the text.

A_e is the measured cumulative amount of intact anthocyanins excreted to the urine, and $A_{e,m}$ is the measured cumulative amount of the sum of anthocyanin metabolites (Eqs. 3 and 4).

$$A_e = f_e \cdot k_a \cdot FD_{po}\left[\frac{1}{k_a} + \frac{e^{-k\cdot t}}{k-k_a} - \frac{k\cdot e^{-k_a\cdot t}}{k_a\cdot\left(k-k_a\right)}\right] \qquad (3)$$

$$A_{e,m} = f_{e,m} \cdot k_m \cdot FD_{po}\left[\frac{1}{k_m} + \frac{e^{-k\cdot t}}{k-k_m} - \frac{k\cdot e^{-k_m\cdot t}}{k_m\cdot\left(k-k_m\right)}\right] \qquad (4)$$

In Eq. (3) f_e is the fraction of absorbed dose excreted into urine, D_{po} the administered dose, k_a the first-order absorption rate constant, k the first-order elimination rate constant and F the unknown bioavailability (assumed to be 1). The model modification arose from switching k_a to k_m, the metabolism rate constant of the sum of metabolites, and $f_{e,m}$ as the fraction of metabolites formed and excreted into urine (Eq. 4).

Using this first-order rate model, the maximum likelihood equation for time of maximum excretion of intact anthocyanins (T_{max}) and sum of metabolites ($T_{max,m}$) may be calculated using Eq. (5) and (6), resp.

$$T_{\max} = \frac{\ln(k_a / k)}{(k_a - k)} \tag{5}$$

$$T_{\max,m} = \frac{\ln(k_m / k)}{(k_m - k)} \tag{6}$$

The elimination half-life for anthocyanins and metabolites is calculated using Eq. (7)

$$t_{1/2} = \frac{0.693}{k} \tag{7}$$

The metabolite ratio R_{met} is calculated using Eq. (8).

$$R_{met} = \frac{A_{e,m}}{A_e} \tag{8}$$

Ranges of starting values were evaluated using results from a preceding non-compartmental PK analysis. The estimated standard errors in each nonlinear regression were determined by the WinNonlin program.

Results and Discussion

The bioanalytical determination of the native anthocyanin content of the QGPJ yielded the administered doses that are summarized in Table I. The ingestion of QGPJ resulted in the appearance of both native QGP anthocyanins and at least five identified anthocyanin metabolites in the volunteers' urine (Figure 1 and 3).

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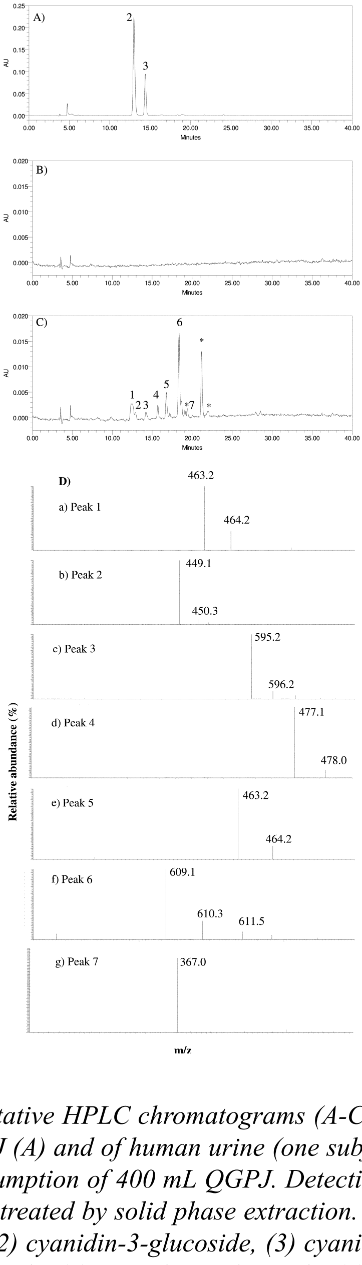

Figure 3. Representative HPLC chromatograms (A-C) and fragmentation patterns (D) of QGPJ (A) and of human urine (one subject) before (B) and 4 h after (C) the consumption of 400 mL QGPJ. Detection was performed at 520 nm. Urine was treated by solid phase extraction. Peaks: (1) cyanidin monoglucuronide, (2) cyanidin-3-glucoside, (3) cyanidin-3-rutinoside, (4) peonidin monoglucuronide, (5) peonidin-3-glucoside, (6) peonidin-3-rutinoside, (7) cyanidin monosulfate, and (*) unknown anthocyanin metabolites. (D) HPLC-ESI-MS precursor scans for product m/z 287 (a-c, g) and m/z 301 (d-f) showing native QGP anthocyanins and metabolites with intact flavylium skeleton. Components are numbered according to (C) (see Appendix D for larger version of figure).

Table I. Administered doses of intact anthocyanins via QGPJ by analysis

	Anthocyanins (mmol)
Cyanidin-3-glucoside	1.84
Cyanidin-3-rutinoside	0.646
Total anthocyanins	2.49

Anthocyanins are calculated as cyanidin-3-glucoside equivalents.

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The results are presented in Table II. Subject 1 excreted 9.5 µmol anthocyanins plus metabolites or 0.38% of the ingested dose, and subject 2 excreted 15.9 µmol or 0.64% of the ingested dose. The two subjects differed in their excretion of intact anthocyanins by 25% (1.8 µmol versus 2.3 µmol), but by 78% in the excretion of anthocyanin metabolites (7.6 µmol versus 13.6 µmol). The first difference could be explained by a difference in bioavailability with an extra variability that was most probably added by different metabolic capacities or physiological state. Cyanidin monoglucuronide and peonidin-3-rutinoside were the primary anthocyanin metabolites identified in the urine samples accounting for 19% (subject 1)/19.6% (subject 2) and 40% (subject 1)/42.5% (subject 2) of the identified anthocyanins and metabolites, respectively. Intact cyanidin-3-glucoside and cyanidin-3-rutinoside accounted for 19.5% (subject 1)/14.6% (subject 2), whereas the sum of the five identified metabolites (cyanidin monoglucuronide, cyanidin monosulfate, peonidin monoglucuronide, peonidin-3-glucoside, and peonidin-3-rutinoside) accounted for 80% (subject 1)/85% (subject 2) of the total anthocyanin excretion.

The metabolite ratio R_{met} (Table III) indicates that the sum of anthocyanin metabolites exceeded the excreted amount of intact anthocyanins 4-fold (subject 1) or 5.7-fold (subject 2).

These results, methylated and glucuronidated derivatives of cyanidin as the main urinary metabolites, are in agreement with several animal and human studies carried out with purified cyanidin compounds, fruit extracts or fruits rich in cyanidins (*8*, *18*). Furthermore, Felgines *et al.* (*16*) demonstrated that with six healthy volunteers after consumption of 200 g of strawberries (with pelargonidin-3-glucoside as the main anthocyanin) more than 96% of the excreted anthocyanins were related to pelargonidin metabolites (predominantly glucuronides). In the present study, the urinary recovery of intact anthocyanins and metabolites was 9.5 to 15.9 µmol corresponding to 0.38 to 0.64% of the administered anthocyanin dose. These data are within the range as reported in the literature for urinary excretion of anthocyanins and metabolites after ingestion of anthocyanin-rich food (reported range: 0.004-5%) (*10*, *18*, *19*).

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Table II. Urinary excretion of anthocyanins and anthocyanin metabolites following ingestion of a single oral dose of 400 ml QGPJ containing 2.49 mmol anthocyanins in two healthy male subjects

Anthrocyanin compounds	*Subject 1*		*Subject 2*	
	$A_{e(0-24)}$ (nmol)	*f_e/f (%)*	*$A_{e(0-24)}$ (nmol)*	*f_e/f (%)*
Cyanidin-3-glucoside	1095	0.0595	1468	0.0797
Cyanidin-3-rutinoside	758	0.117	854	0.132
Intact anthocyanins1	**1854**	**0.0745**	**2322**	**0.0933**
Cyanidin monoglucuronide	1824	0.0733	3112	0.125
Peonidin monoglucuronide	1504	0.0605	2467	0.0991
Peonidin-3-glucoside	226	0.0091	1058	0.0425
Peonidin-3-rutinoside	3772	0.152	6742	0.271
Cyanidin monosulfite	279	0.0112	176	0.0071
Sum metabolites2	**7606**	**0.306**	**13555**	**0.545**
Sum intact anthocyanins and metabolites	9460	0.38	15877	0.64

Results are expressed as cyanidin-3-glucoside equivalents. $A_{e(0-24)}$: amount of compound excreted into urine within 0 to 24 hours, f_e/f: fraction of orally administered compound excreted into urine within 24 hours (for the glucuronides, sulfates, peonidin glycosides, and the sums f_e/f is related to 2.49 mmol intact anthocyanins). [1] Sum of cyanidin-3-glucoside and cyanidin-3-rutinoside [2] Sum of cyanidin monoglucuronide, peonidin monoglucuronide, peonidin-3-glucoside, peonidin-3-rutinoside and cyanidin monosulfate

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Table III. Primary model parameter estimates and derived, secondary PK parameter

Model parameter	*Subject 1*		*Subject 2*	
	Estimate	*Standard error*	*Estimate*	*Standard error*
Intact anthocyanins				
k_a (h^{-1})	14.5	96.3	1.89	6.98
k (h^{-1})	0.42	0.092	0.48	0.64
f_e	0.000754	0.00002	0.000957	0.0001
T_{max} (h)	0.25	1.24	0.97	1.74
$T_{1/2}$ (h)	1.67	0.37	1.45	1.93
A_e (nmol)	1876	48.5	2382	329
r^1	0.9996		0.9987	
Sum metabolites				
k_m (h^{-1})	1.58	0.208	0.55	1.94
k (h^{-1})	0.28	0.012	0.60	2.21
$f_{e,m}$	0.00303	0.00002	0.00549	0.00016
$A_{e,m}$ (nmol)	7547	59.9	13658	396
T_{max}	1.33	0.095	1.74	0.26
$T_{1/2}$ (h)	2.46	0.102	1.16	4.27
R_{met}	4.02	0.109	5.73	0.808
r	0.9996		0.9959	

Secondary parameters are A_e, $A_{e,m}$, T_{max}, $T_{1/2}$ and R_{met}. A_e, and $A_{e,m}$ are predicted amounts because they are calculated from the estimated model parameter f_e ($f_{e,m}$) × Dose. [1] Correlation coefficient between observed and predicted amounts.

The cumulative excretion of anthocyanins could be fitted to a one-compartment model with instantaneous, parallel excretion of anthocyanin metabolites (Figure 4).

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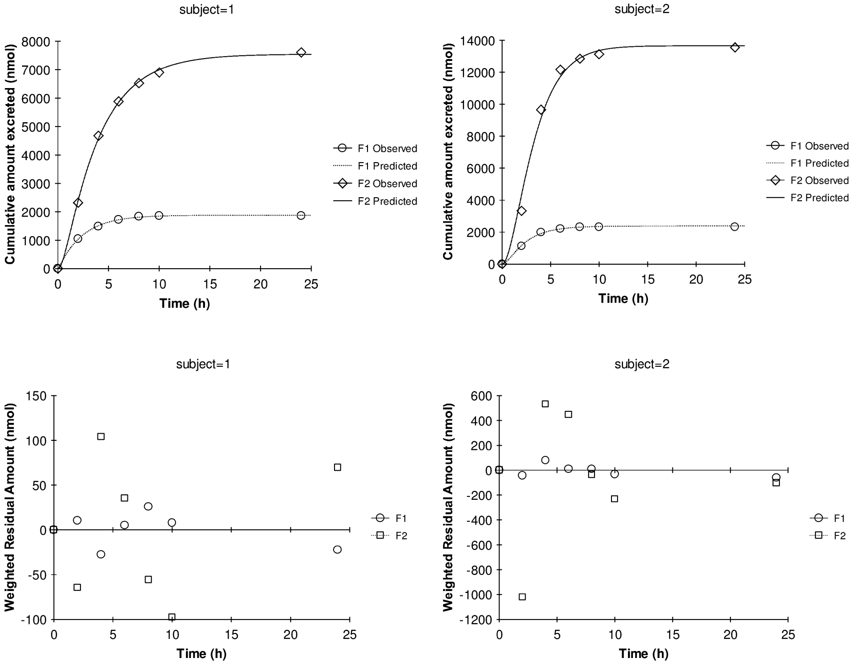

Figure 4. Cumulative amount of intact anthocyanins (F1) and sum of anthocyanin metabolites (F2) excreted by the urine and the predicted cumulative renal excretion. A one-compartment model with parallel (parent/metabolite) first-order renal elimination was fitted to data. Lower panel: goodness-of-fit plots indicating a satisfying fit, i.e., residuals are evenly spread around the zero value over time, of intact anthocyanins (F1), but a problematic fit of the sum of anthocyanin metabolites (F2), especially for subject 2.

T_{max} of intact anthocyanins was 0.25 h (subject 1) and 0.97 h (subject 2, Table III). However, the absorption rate constant k_a, and consequently T_{max} for intact anthocyanins, could only poorly be determined as the large standard error indicates. T_{max} of the metabolites was 1.33 h (subject 1) and 1.74 h (subject 2), respectively. The rapid peak excretion of the native cyanidin glycosides within 2 h after QGPJ consumption appears to confirm the important role of gastric absorption. In addition, the formation of anthocyanin metabolites is characterised as a rapid process. The metabolism rate constant k_m and the elimination rate constant of the metabolites k could be determined precisely in subject 1, but less precisely in subject 2. The reason is a large correlation of k and k_m (>0.95) in subject 2 (results not shown) which simply reflects that these rate constants are very close in magnitude.

The inspection of the plots of the fitted curve superimposed on the observed data and the residuals over time indicates that the consistency was more or less satisfying between observed and predicted data for the proposed model (Figure 4). In addition, the correlation coefficient r reached more than 0.995 for both subjects (Table III).

The half-life of both intact anthocyanins and metabolites is short (<2.5 hours). Under the assumption of the high k_a (rapid absorption) and short half-life of anthocyanins the poor precision of the estimates can also be caused by inappropriate design, i.e., urine collection intervals. Study design should be optimized such that the bin width of urine collection intervals is smaller than 2 hours at early post-dose time points. The non-linear modeling analysis is suitable to use spot urine samples collected at irregular, but early time points (according to the natural urinating scheme) in a more precise estimation of pharmacokinetic parameters.

The maximal excretion of cyanidin-3-glucoside and cyanidin-3-rutinoside was observed during the first 2 h (~52% of total anthocyanin excretion) whereas the metabolites' excretion was maximal between 2 and 4 h (~41% of total metabolite excretion) after QGPJ consumption. It is apparent that the urinary excretion of peonidin-3-rutinoside continued until the end of the experimental treatment (24 h post ingestion). A similar excretion pattern was observed by Felgines et al. (*16*) after the consumption of strawberries rich in pelargonidin-3-glucoside by six healthy volunteers: the maximal excretion of intact pelargonidin-3-glucoside was observed during the first 2 h, whereas the maximal excretion of pelargonidin metabolites was measured between 2 and 4 h after the strawberry breakfast. The rapid peak excretion of the native cyanidin glycosides within 2 h after QGPJ consumption appears to confirm the important role of gastric absorption. After absorption from the stomach into the bloodstream the native QGP anthocyanins are glucuronidated and methylated through the activities of UDP-glucuronyl-transferase and catechol-O-methyltransferase, respectively, mostly in the liver and kidney which are the major sites of glucuronidation and methylation *in vivo* (*18*). Besides glucuronidated and methylated derivatives, Felgines and colleagues could also detect sulfoconjugates of pelargonidin and cyanidin in the volunteers' urine after the consumption of strawberries and blackberries, respectively (*16*, *20*). The urinary recovery of cyanidin monosulfate in the blackberry study was too low for an unambiguous identification on the HPLC-DAD chromatogram and subsequent quantification (*20*); whereas in the strawberry study, pelargonidin monosulfate could be quantified in the volunteers' 24 h urine in the same concentration range (>100 nmol) as pelargondin-3-glucoside (*16*). The results of the latter study are in agreement with our findings: both subjects excreted significant amounts of cyanidin monosulfate (279 nmol (subject 1)/176 nmol (subject 2)) after QGPJ ingestion. It is remarkable, that cyanidin monosulfate is the only analyte that subject 2 excreted less than subject 1 (Table I). Sulfoconjugate formation requires hydrolysis (e.g. by lactase phloridzin hydrolase) of the glycosides to the respective aglycons (anthocyanidins) and then sulfoconjugation of the aglycons by sulfotransferases which is present in the intestine, liver, and other tissues (*11*, *20*). However, no aglycons could be detected in the present study with QGPJ

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whereas Felgines and colleagues reported significant amounts of pelargonidin as well as cyanidin and peonidin in the volunteers' 24 h urine after the ingestion of strawberries and blackberries, respectively (*16*, *20*). Due to the instability of anthocyanin-derived aglycons at physiological pH, the authors suggested that the detected aglycons are most likely the product of β-glucosidases and sulfatases present in kidney tissue and urine (*21*, *22*) and do not arise from the small intestine. But several other bioavailability studies failed to detect aglycons in plasma and/or urine of human subjects after the consumption of anthocyanin-rich food, which is consistent with our results (*8*, *9*, *23–25*).

At baseline, and in the control treatment, all urine samples did not contain any native anthocyanins, aglycons or metabolites with intact flavylium skeleton.

The degradation of dietary anthocyanins to several phenolic acids, such as homovanillic, vanillic, protocatechuic, p-hydoxybenzoic, and syringic acids by *in vitro* models mimicking fecal fermentation as well as in *in vivo* studies with healthy human subjects has been demonstrated recently (*1*, *18*, *26–30*). Furthermore, Woodward and colleagues showed that anthocyanins rapidly degraded to their respective phenolic acid and aldehyde constituents under simulated physiological conditions (*31*). A single anthocyanin may generate several main and minor metabolites, perhaps as many as 20 as in the case of quercetin glycosides (*32*) or even more. It is obvious that the compounds that reach the cells and tissues are chemically and biologically different from the dietary forms in the plants/plant food and that such features may have a significant effect on their potential bioactivity *in vivo*.

The anti-thrombotic activities of dihydroferulic acid and 3-(3-hydroxyphenyl)propionic acid, both putative colonic metabolites of cyanidin and delphinidin based anthocyanins, are an example for the biological significance of these catabolites (*33*). Although the identification and quantification of anthocyanin metabolites represents an emerging field of research since the interest about the potential impact of these plant pigments on health and disease prevention has significantly increased over the past decade, the potential biological activity of anthocyanin metabolites needs to be better investigated (*1*, *7*).

Consequently, it would be highly desirable in future *in vivo* studies with animals and/or human subjects to utilize labelled anthocyanins for the identification of all major metabolites which should include conjugates with intact flavylium skeleton as well as catabolites produced by the intestinal microflora. Otherwise, the 'true' bioavailability of anthocyanins and their relevance for human health and disease prevention will remain a 'Black-Box'.

Conclusion

This is the first published report about the urinary pharmacokinetics of QGP anthocyanins and metabolites in healthy human subjects. The urinary excretion of QGP anthocyanins, mainly as glucuronidated and methylated metabolites within 24 h after QGP juice consumption by two healthy male subjects, indicates an extensive metabolism of the native QGP anthocyanins.

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The usefulness of non-linear modeling statistical techniques for characterizing the urine excretion-time profiles and estimating relevant PK parameters has been shown. This statistical technique, coupled with experimental designs in which multiple spot urine voids are collected for a specified period after a short-term (peak or spike) exposure, is useful for the determination of maximum excretion time, a potentially important parameter in bio-monitoring under non-steady-state conditions. Blood samples should be used in future studies, as the benefits of simultaneously utilizing two different sources of data for modelling in regards to the increase in parameter precision has been demonstrated repeatedly. Thus, the potential biological activity of the metabolites derived from QGP anthocyanins (including catabolites produced by the intestinal microflora) needs to be better investigated in the future. Therefore, additional human studies with QGP and/or derived products including blood plasma, urine and fecal samples, as well as a larger number of subjects, are warranted to identify the complete spectra of *in vivo* metabolites and to investigate their interaction with chronic disease processes by using appropriate cell-based assays and animal models.

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Chapter 22

Conjugated Fatty Acids as a Prevention Tool for Obesity and Osteoporosis

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Since the identification of conjugated linoleic acid (CLA) as an anticarcinogen from beef in the 1970s, it has been studied for a wide range of biological activities, including reducing development of atherosclerosis, enhancing animal growth, modulating immune responses, and interestingly reducing body fat while enhancing lean body mass. It is suggested that the variety of biological activities of CLA may be due to two main isomers, *cis*-9,*trans*-11 and *trans*-10,*cis*-12. In addition to CLA, other cognates of CLA have been tested for their bioactivities. Among them, a 19-carbon cognate of CLA, conjugated nonadecadienoic acid (CNA), showed greater efficacy on body fat reduction in an animal model, where CLA and CNA share biochemical mechanisms. CLA has also shown potential to improve bone mass. Thus conjugated fatty acids have great potential to be used to prevent obesity and osteoporosis in conjunction with currently available treatments.

Introduction

Conjugated linoleic acid (CLA) is a group of geometric and positional isomers of linoleic acid. CLA was identified in the 1930s, however, its bioactivities were first discovered as an anticancer component from ground beef in the 1980s (*1*, *2*). Since then, considerable research discovered a wide range of biologically beneficial effects of CLA, such as reducing a number of types of cancer, controlling atherosclerosis development, modulating immune responses, reducing body fat, and improving bone mass (*3*, *4*). In particular, the effects of CLA on body fat

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drew significant interest for use of CLA to prevent obesity (*3*, *4*). Moreover there is significant interest in the effects of CLA with regard to osteoporosis prevention. As CLA has been approved as generally recognized as safe (GRAS) for use in certain types of food since 2008 in the US, there is great need to understand the mechanisms of CLA on health effects. This chapter will focus on the mechanisms as well as potential applications of CLA for controlling obesity and osteoporosis.

Origins and Isomers of CLA

CLA is primarily found in beef, dairy products (milk, cheese, cream, butter, or yogurt), and meats from ruminant origin (*5*). This is because CLA originates as an intermediate product during the biohydrogenation process of linoleic acid to stearic acid by rumen bacteria (*6*). The main isomer found in food is the *cis*-9,*trans*-11 isomer, where 80-90% of CLA in food is this form (*5*). Alternatively, the *cis*-9,*trans*-11 CLA isomer can originate by delta-9 desaturation of *trans*-11 vaccenic acid in mammalian tissue, which is also an intermediate for the biohydrogenation process and the most abundant *trans* fatty acids in natural sources (*7–9*).

Among a number of other geometric and positional CLA isomers that have been reported, the other main CLA isomer of interest is the *trans*-10,*cis*-12 isomer (*3*, *10*, *11*). This isomer is found at very low levels in foods, however, when CLA is prepared synthetically from linoleic acid, this along with the *cis*-9,*trans*-11 isomer make up about 85-95% of total CLA, with approximately a 1:1 ratio of these two isomers, often called '50:50 mixture' (*5*, *12*). Most biological activities of CLA are suggested to be the result of interactions between these two isomers, *cis*-9,*trans*-11 and *trans*-10,*cis*-12 (*3*, *10*, *11*). Thus this chapter will focus on their bioactivities.

Although the two major CLA isomers include a '*trans'* configuration, it is important to note here that CLA is excluded from bing a '*trans'* fat in the Nutrition Facts labeling. The '*trans'* fat from the Nutrition Facts labeling includes non-conjugated *trans* fats from food, originated either from partially hydrogenated vegetable oils or from natural sources, such as ruminant origin (*13*, *14*). This is based on the significant difference in biological functions between non-conjugated and conjugated *trans* fats, mainly CLA (*3*, *10*, *13*, *14*).

Antiobesity Effects of CLA

CLA has been shown to control body fat, which has drawn considerable interest (*3*). In mouse models, CLA effectively and consistently reduced total body fat, in some instances over 50% reduction of body fat compared to control (*15*, *16*). At the same time, the lean mass as represented as total protein was significantly increased in these animals (*15*, *16*). Earlier studies mainly focused on controlling fat accumulation by using relatively young fast growing animals to study CLA's effects (*3*, *16*), however, there are reports using older mice where CLA also effectively reduced body fat in these models, suggesting CLA reduces existing fat as well (*3*, *17*). Among the two major isomers, the *trans*-10,*cis*-12 CLA isomer has been linked, while the *cis*-9,*trans*-11 isomer has no contribution, to CLA's antiobesity effect (*16*, *18*).

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Mechanisms of Antiobesity Effects by CLA

Multiple mechanisms of action for CLA's control of body fat have been suggested; increasing total energy expenditure, reducing fat accumulation and/or adipocytic differentiation, increasing fatty acid β-oxidation in skeletal muscle, and modulating adipokines and cytokines (*3*, *16*).

First, CLA has been shown to increase total energy expenditure, as shown in both animal and human studies (*19–26*). However, some human studies failed to show any significant effects of CLA on energy expenditure, even with reduced body weight or fat deposition (*27–30*). Secondly, CLA has significant influence on adipocytes; reducing fat uptake, reducing lipogenesis, increasing apoptosis, inhibiting preadipocytic differentiation, and increasing lipolysis, as supported by both *in vitro* and *in vivo* studies (*4*, *15*, *16*, *18*, *31–35*). Thirdly, CLA increased fatty acid β-oxidation in skeletal muscle, as shown by decreased respiratory quotient and increased carnitine palmitoyl transferase I expression and/or activity (*26*, *36–41*). Lastly, CLA has been linked with modulation of adipokines and cytokines, such as leptin, tumor necrosis factor-α, or adiponectin, which may be involved in controlling food intake and satiety (*42–44*). Thus, all of these mechanisms may play roles in how CLA effectively reduces body fat.

Species Specificity for CLA's Obesity Control

Response to CLA with regard to body fat control was most pronounced in the mouse model, while other species, including humans, responded with less body fat reduction (*3*, *45–50*). These differences may be related to dose administered, duration of study, differences in metabolic rates amongst species, and differences in experimental design (*3*, *3*, *16*, *47*, *51*).

Previous CLA studies with mice used diets containing 0.5 w/w% CLA, which is equivalent to about 56g CLA/day/70 kg (*52*). In comparison, most human studies published used 0.7-6.8g CLA per day, which is substantially lower than doses used in mice. Secondly, a 4-week CLA feeding period is used for most mice studies. In comparison, human clinical trials with 4 weeks supplementation or shorter did not observe any effects of CLA on body fat reduction (*3*). However, in human studies of longer than 6 months duration, CLA has shown significant reduction in body fat (*3*, *45*). Alternatively, differences in metabolism between species may provide the explanation of differing effects, as mice have a higher fat turnover rate and energy requirement per unit of body weight than other species (*51*).

Another important possibility is the difference in dietary regimes; ad libitum in animal models (a positive energy balance) compared to dietary restriction in human trials (negative energy balance). It was previously reported in a mouse study that antiobesity effects of CLA were not observed during dietary restriction (negative energy balance) (*17*). Similarly, there was consistent observation that CLA had no effects on body fat in clinical studies, when a hypocaloric diet plan (particularly greater than 200 Cal restriction per day) was used regardless of dose or duration of CLA supplementation (*3*, *44*, *53*, *54*). This suggests that CLA may be most effective at reducing fat mass gain, particularly in humans, as shown previously (*19*, *21*, *42*, *44*, *53*, *55*).

Application of CLA for Prevention of Osteoporosis

Along with the observation that CLA reduces body fat, previous publications reported improved bone mass after CLA supplementation (*3*, *49*, *56*, *57*). However, others reported inconsistent observations regarding CLA's effect on bone health, in particular bone mineral content or bone mineral density in animal models (*56–58*). Currently, there are two human studies reporting beneficial effects of CLA on bone health (*59*, *60*), while others do not report such an effect (*61–63*).

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CLA and Calcium Interaction

Recently, we reported that the inconsistent effects of CLA on bone health may be due in part to the interaction between dietary CLA and calcium levels (*57*). CLA supplementation with additional calcium in the diet improved total ash weights as an indicator for total bone mass in mice (*57*). In fact, Brownbill *et al.* (*59*) reported a benefit of CLA intake among postmenopausal women consuming a calcium supplement. It is also important to point out that this study estimated CLA intake from dietary records, thus the major CLA isomer linked to this study was the *cis*-9,*trans*-11 isomer, not the *trans*-10,*cis*-12 isomer. The potential role of the *trans*-10,*cis*-12 CLA isomer along with calcium in humans is not clear at this moment.

Mechanisms of CLA's Effect on Bone

Compared to extensive research on CLA's mechanisms on body fat reduction, there is currently limited studies on mechanisms of CLA on bone health. As suggested in body fat reduction, multiple mechanisms of CLA on bone mass have been suggested; increasing calcium absorption, promoting bone formation, and suppressing bone resorption.

First, it has been reported that CLA improved calcium absorption from the intestines (*64–69*). CLA increased calcium transport by involving transcellular and paracellular pathways in the Caco-2 human colon adenocarcinoma cell line (*64–68*). However, inconsistent results were observed with the two major CLA isomers' effects on calcium transport (*64–68*). The exact mechanism of these two CLA isomers on calcium transport as well as CLA's influence on hormones for calcium homeostasis needs further investigation.

Secondly, CLA may influence bone remodeling by shifting to greater bone formation and less bone resorption. Bone remodeling is the process that occurs throughout life of bone formation by osteoblasts and/or bone resorption by osteoclasts (*70*). Osteoblasts originate from bone marrow mesenchymal stem cells, while osteoclasts originate from macrophage lineage hematopoietic stem cells (*71*, *72*). Bone marrow mesenchymal stem cells can also differentiate into bone marrow adipocytes, where a negative correlation exists between bone marrow adipocytes and bone formation (*71–74*). Based on the observation that CLA reduces adipocytic differentiation, CLA has potential to improve bone mass by controlling bone marrow adipocytes (*10*, *16*). It has been consistently observed that CLA significantly improves bone mass only when CLA reduces body fat in the same animals (*15*, *18*, *31*, *36*, *75–84*). This is supported further by the evidence that *trans*-10,*cis*-12 CLA, the active isomer for body fat reduction, is also responsible for improving bone mass (*56*). In fact, it has recently been reported that CLA indeed improves bone formation while decreasing bone adiposity in mesenchyme stem cells and animal models (*83–85*). Alternatively, CLA directly influences bone resorption by osteoclasts. CLA has been shown to reduce the markers of osteoclastogenesis ((*83*, *85*, *86*) and unpublished observation). Although there are still inconsistencies with regard to CLA's effects on bone resorption, CLA is quite a promising dietary component for potentially improving bone mass and thus reducing osteoporosis.

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Other Conjugated Fatty Acids

To understand the key molecular structures for CLA's bioactivities as well as to improve the efficacy of CLA's body fat reduction in humans, a number of fatty acids, including novel conjugated fatty acids, were tested (*26*, *31*, *80*, *82*).

Structure-Activity Relationships

Various mono-unsaturated octadecenoic acids were tested for their ability to inhibit lipoprotein lipase (LPL) activity in adipocytes, the results of which are summarized in Table 1. Inhibition of adipocytic lipoprotein lipase activity has been well correlated with the ability of these fatty acids to reduce body fat in the mouse model (*31*). Based on the results of various 18-carbon fatty acids, it was concluded that conjugated double bonds are required for CLA's activity on body fat regulation.

Other Conjugated Fatty Acids

Conjugated fatty acids with different carbon-length were also tested, which include 19-, 20-, and 21-carbon conjugated fatty acids (Table 1). Among them, 19-carbon conjugated fatty acids, conjugated nonadecadienoic acids (CNA), inhibited LPL activity and further study indicated that CNA reduced body fat more effectively than CLA (*26*, *80*). Meanwhile, 20-carbon conjugated fatty acids, conjugated eicosadienoic acids (CEA), inhibited LPL activity although they were less active than CLA (*82*). In the biological system CEA reduced body fat by converting to CLA as shown in animal studies using the mouse model (*82*). 21-carbon conjugated fatty acids, heneicosadienoic acids (CHDA), did not have any effects on lipoprotein lipase activity in adipocytes, which suggests no effects on body fat in an in vivo model (*82*). In addition, an elongated and desaturated form of CLA, *cis*-8,*trans*-12,*cis*-14 eicosatrienoic acid, did not have any effect on lipoprotein lipase activity. This suggests that only CLA and CNA, but not other fatty acids including conjugated fatty acids tested, are linked to body fat reduction (*31*, *82*). It has been recently reported that CNA exerts its effect on body fat regulation through mechanisms similar to those of CLA; increased energy expenditure and fatty acid β-oxidation (*26*). The improved efficacy of CNA on body fat reduction compared to CLA has been suggested to be linked to its ability to enhance lipolysis in adipocytes (*26*).

Table 1. Effects of fatty acids on adipocytic lipoprotein lipase activity[1]

Fatty acids	*Inhibition of LPL[2] Activity*
18-carbon fatty acids	
Mono-unsaturated	
cis-9 (oleic)	-[2]
cis-11	-
cis-12	-
cis-13	-
trans-9	-
trans-10	-
trans-11	-
trans-12	-
trans-13	-
Poly-unsaturated	
cis-9,*cis*-12 (linoleic acid)	-
trans-9,*cis*-12	-
trans-10,*cis*-12 CLA[2]	++[2]
cis-9,*trans*-11 CLA	-/+
trans-9,*trans*-11 CLA	-
19-carbon fatty acids	
cis-10 nonadecenoic acid	-
trans-10 nonadecenoic acid	-
cis-10,*cis*-13 nonadecadienoic acid	-
cis-10,*trans*-12/ *trans* 11-,*cis*-13 CNA[2]	++
20-carbon fatty acids	
cis-11,*cis*-14 eicosadienoic acid	-

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Table 1. (Continued). Effects of fatty acids on adipocytic lipoprotein lipase activity[1]

Fatty acids	*Inhibition of LPL[2] Activity*
cis-11,*trans*-13 and *trans*-12,*cis*-14 CEA[2]	+
cis-11,*trans*-13 CEA	-
cis-8,*cis*-11,*cis*-14 eicosatrienoic acid	-
cis-8,*trans*-12,*cis*-14 eicosatrienoic acid	-
21-carbon fatty acids	
cis-12,*cis*-15 heneicosadienoic acid	-
cis-12,*trans*-14/ *trans*-13,*cis*-15 CHDA[2]	-

[1] Data are summarized from (*26*, *31*, *80*, *82*). [2] -, no change; +, inhibition; LPL, lipoprotein lipase activity; CLA, conjugated linoleic acid; CNA, conjugated nonadecadienoic acid; CEA, conjugated eicosadienoic acid; CHDA, conjugated heneicosadienoic acid.

Other CLA Metabolites

In addition to being metabolized by elongation and/or desaturation, CLA is reported to be subjected to fatty acid β-oxidation (*4*, *82*). Reported metabolites of CLA generated by fatty acid β-oxidation are conjugated hexadecadienoic (conj. $\Delta16:2$), tetradecadienoic (conj. $\Delta14:2$), and dodecadienoic (conj. $\Delta12:2^{c3,t5/t4,c6}$) acids (*82*, *87–90*). Currently none of these CLA metabolites has been tested for activities.

Potential Concerns

Safety concerns associated with CLA uptake are fatty liver, milk fat depression, glucose tolerance, and increased oxidative markers (*3*, *4*, *10*).

There were pronounced effects of fatty liver in mouse studies with CLA. This coincided with tremendous lipid mobilization from adipose tissue and increased hepatic fatty acid synthesis (*91*, *92*). Animal studies suggest that effects of CLA on the liver may be transient and reversible responses (*93*, *94*). Human clinical trials reported minimal changes in serum markers for liver functions (*21*, *44*, *60*, *95*, *96*). However, there was a report of a single case of CLA induced acute hepatitis, thus there is need for careful determination regarding CLA supplementation and possible liver toxicity (*97*).

CLA also decreased milk fat content in cow's milk (*98*, *99*). Thus concerns over lactating humans were raised. However, studies showed no effect of CLA on milk fat content in humans, but human studies used a relatively short supplementation period, less than 5 days, to draw any conclusion for CLA's effect on human milk fat reduction (*100*, *101*).

Inconsistent effects of CLA on glucose metabolism have been reported in both animals and humans (*3*, *4*, *29*, *94*, *102–107*). It is suggested that

CLA modulates glucose metabolism through changes in fatty acid β-oxidation, cytokines, adipokines, and/or glucose uptake (*108–115*). However, it is important to note that human studies with longer than 6 months of CLA supplementation reported no changes in glucose or insulin levels and insulin sensitivity (*21*, *44*, *53*, *60*, *62*, *95*, *96*, *106*, *116*, *117*). Thus influence of CLA on glucose metabolism may be a transient effect (*3*).

There are consistent observations of increased oxidative markers after CLA supplementation from human studies (*20*, *118–125*). This is associated with both *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers (*118–122*). The significance of this observation is not clear at the moment and needs careful evaluation in the near future.

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Conclusion

Although there are safety concerns associated with CLA supplementation, CLA has great potential to be used along with other available supplements or treatments for obesity. In addition, CLA has been shown to be beneficial for bone health. With current GRAS status of CLA in the US, there are great opportunities to use CLA to improve health as well as challenges for evaluating CLA's safety issues.

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Chapter 23

NMR Spectroscopy-Based Metabolic Profiling for Detecting Hepatobiliary Diseases

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The emerging area of metabolomics deals with the detection of a large number of small molecules (metabolites) from human biofluids or tissue in a single step and promises immense utility for early diagnosis, therapy monitoring and understanding pathogenesis of many diseases. Metabolomics methods are focused on the information rich analytical techniques of nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). While MS is highly sensitive, NMR spectroscopy provides more reproducible and quantifiable data. Focused on the development of simple NMR based tools for metabolomics applications, new NMR methodologies have been explored. The methods were applied to detect hepatobiliary disease biomarkers using human bile, which is closely associated with the pathological source. Causes or consequences of numerous diseases including hepatocellular carcinoma, cholangiocarcinoma and gallbladder cancer have been attributed to the altered human bile metabolic profile.

Profiling of small molecules (metabolites), in biological samples such as blood, urine, bile, saliva, cerebrospinal fluid and tissue specimens, which are the downstream products of genes, mRNAs and proteins, promises numerous clinical applications including early disease diagnostics (*1–4*). The significant interest in metabolite profiling arises from the high sensitivity of metabolites to subtle changes in the biological state. Such high sensitivity of metabolites is valuable for early detection of onset of many diseases that are normally asymptomatic until late in the disease process. An important advantage of metabolite profiling

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over other approaches is that a large number of metabolites can be analyzed quantitatively and reproducibly using advanced analytical techniques. Further, this approach is either non- or minimally-invasve and requires minimal or no sample preparation steps. These characteristics are very important and attractive for translating the findings to clinical applications.

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the most powerful analytical techniques used for metabolic profiling (*2*). NMR spectrosocpy offers numerous advantages because of its ability to provide accurate and reproducible information on a large number of metabolites, in a single-step and using intact samples. However, it is challenging to obtain NMR spectra for biological samples with well resolved peaks from a single experiment, and the knowledge of identity of a large number of metabolites in complex NMR spectra is lacking. The high complexity to the NMR spectra arises due to the presence of thousands of metabolites in the biological samples. Such vast chemical diversity and the concentration range of over eight orders of magnitude (*5*) render detection of all the metabolites from a single analytical technique impractical Hence novel analytical methods are required to analyze complex biological mixtures.

Advanced NMR Methods for Metabolite Profiling

To circumvent the challenges associated with high complexity of the biological samples, we focused on the development of new NMR methods targeting detection of individual classes of metabolites based on their chemical functional groups. Specifically, metabolites containing amino, carboxyl and hydroxyl functional groups were targeted individually by tagging with isotopes such as ^{13}C, ^{15}N or ^{31}P. One-dimensional (1D) or two-dimensional (2D) NMR experiments involving these heteronuclei selectively detect isotope labeled metabolites with greatly improved resolution and sensitivity, free of background signals from the rest of the tagged molecule as well as the untagged molecules (*6–9*).

Tagging of the amine class of metabolites involves a simple chemical reaction on intact biological samples using ^{13}C labeled acetic anhydride. A subsequent ^{1}H-^{13}C 2D HSQC (heteronuclear single quantum coherence) NMR experiment detects amine class of metabolites with improved sensitivity and resolution (*6*). More recently, we introduced an alternative method, which involves isotope tagging using ^{13}C labeled formic acid to further improve the performance of ^{13}C isotope tagging experiments (*7*). This method is more robust than the earlier method since the short one-bond distance between the labeled ^{13}C and its closest ^{1}H produces a large *J*-coupling constant and enables efficient transfer of polarization between ^{13}C and ^{1}H using shorter interpulse delays in the 2D experiments. Furthermore, the close distance between the isotope tag and metabolite leads to somewhat wider dispersion of the tagged metabolites' signals in the resultant 2D spectrum.

Tagging carboxyl group containing metabolites in biological samples involves derivatization with ^{15}N labeled ethanolamine. The ^{1}H-^{15}N 2D HSQC NMR experiment then selectively detects the ^{15}N tagged carboxylic acids in a single measurement with enhanced resolution and sensitivity (*8*). This method exploits the broad chemical shift dispersion of ^{15}N nuclei and imparts a large dispersion to the individual metabolites signals. This approach, enables detection of nearly two hundred carboxyl group containing metabolites in biological samples, both quantitatively and reproducibly. Detection of such a large number of metabolites is unprecedented from the NMR spectroscopy point of view (Fig. 1).

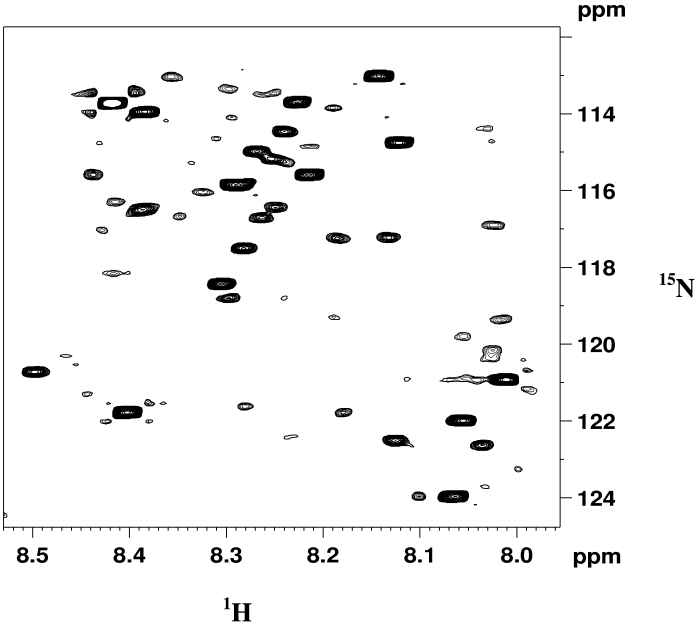

Figure 1. ^{1}H-^{15}N 2D HSQC NMR spectrum showing the detection of carboxylic acid class of metabolites using ^{15}N isotope tagging (8).

Isotope tagging of hydroxyl group containing metabolites involves derivatization of metabolites using an agent containing ^{31}P, 2-chloro-4,4,5,5-tetramethyldioxaphospholane (*9*). ^{31}P tagging approach also detects metabolites containing aldehyde and carboxyl groups. ^{31}P tagged metabolites are detected with enhanced resolution using one-dimensional ^{31}P NMR by exploiting the 100% abundance and wide chemical shift range of ^{31}P NMR spectrum.

All the isotope labeling approaches that we have developed are simple, highly reproducible and quantitative. Metabolites containing amino, carboxyl and hydroxyl functional groups constitute major classes of metabolites in biological processes. Hence, our ability to detect and quantify them using the new isotope tagging approaches represents a significant step forward in the area of metabolites based biomarkers detection for a number of diseases.

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Human Blood and Urine

For a number of reasons human blood plasma/serum and urine have been extensively used in the area of metabolomics. Urine can be acquired non-invasively and it provides several hundreds of detectable signals even by relatively less sensitive analytical methods such as NMR spectroscopy. Blood, on the other hand, maintains normal metabolic homeostasis and any changes to this status represent a pathological state. However, the complexity due to numerous confounding factors such as age, gender, diet, body mass, lifestyle, drug, environment and diurnal variation, and pathological conditions involving multiple organs poses a significant challenge to detect biomarkers for disease of interest using human urine and blood plasma/serum samples. To overcome such challenges, metabolic profiling efforts were focused on other biological specimens such as human bile and tissue. A growing number of studies suggest that disease biomarkers are richly populated in such specimens due to their close association with the pathological source (*10–13*).

Human Bile as a Source of Hepatobiliary Disease Biomarkers

Human bile is a complex fluid synthesized and secreted by the liver (*14*), and transported through bile canaliculi and bile ducts, and stored in the gallbladder. The bile gets concentrated in the gallbladder and emptied into the small intestine (duodenum) upon ingestion of food. Bile constitutes a mixture of organic molecules such as cholesterol, phospholipids, bile acids, urea, glucose, steroid hormones and bilirubin, and a variety of proteins and peptides. Under normal conditions, the constituents of bile are tightly controlled through homeostasis. Alterations in normal metabolism due to etiological conditions of hepatobiliary system represent a novel source of liver disease-specific biomarkers (*12*). Since the bile constitutes a pool of metabolites closely associated with the gastrointestinal system, metabolic profile of bile closely represents normal or abnormal cellular processes associated with the liver, bile ducts and gallbladder. Hence analysis of bile provides valuable clues to potential biomarkers associated with numerous hepatobiliary diseases. Among a large number of bile metabolites, cholesterol, glycerophospholipids and bile acids represent the most abundant constituents of human bile. Bile acids constitute a group of a large number of structurally closely related molecules, which are synthesized starting from cholesterol and conjugated mainly with glycine or taurine before secretion from the hepatocytes (*15*, *16*).

Metabolite Profiling of Human Bile Using NMR Spectroscopy

Severe overlap of signals from a large number of bile metabolites, which exist in aggregated form because of amphipathic nature of the molecules, is a major challenge in the analysis of human bile using NMR spectroscopy. To circumvent this problem, numerous advanced NMR methodologies have recently been applied and unraveled the complexity of human bile spectrum (*17–22*). This work involved extensive analysis of synthetic compounds and establishment of a library of ^{1}H and ^{13}C NMR chemical shifts for numerous bile metabolites under physiological conditions (*22*). Subsequently, using a combination of the chemical shift library, and homonuclear and heteronuclear NMR experiments at 400, 700 and 800 MHz, comprehensive analysis of bile NMR spectra was made. An important outcome of this study is the identification of all major bile metabolites including bile acids, cholesterol, phospholipids, unsaturated lipids and urea, all of which are known to be commonly found in healthy human bile (*18*, *19*). In human bile, six conjugated bile acids are commonly present at high concentration. These constitute two primary bile acids, cholic acid and chenodeoxycholic acid, and a secondary bile acid, deoxycholic acid. Both primary and secondary bile acids are conjugated to glycine, resulting in glycocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid, and to taurine, resulting in taurocholic acid, taurodeoxycholic acid and taurochenodeoxycholic acid. We identified all these six bile acids individually in bile for the first time from the point of view of NMR. Subsequently, simple NMR methods were developed for high-throughput analysis of all major bile metabolites in a single step for routine applications.

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Visualization of Human Bile Homeostasis for Assessing Hepatobiliary Diseases

Concentrations of biliary metabolites are regulated through homeostatic control. Any alterations in metabolic conditions arising from etiological conditions of hepatobiliary diseases, including alterations in the bile synthesis by the liver or enterohepatic circulation, represent a novel source of liver disease-specific biomarkers. Since the bile constitutes a pool of both endogenous and exogenous compounds excreted from the cells of the liver, gallbladder and bile ducts, the metabolic profile of bile closely represents normal as well as abnormal cellular metabolic processes associated with these organs, and hence investigations of bile composition potentially provide valuable clues to the biomarkers of hepatobiliary diseases for diagnostic and therapeutic applications. Recent investigations of bile using single-step NMR methods showed significant alterations in bile synthesis and enterohepatic circulation for a number of hepatobiliary diseases, including malignancies. Cholangiocarcinoma and non-malignant liver diseases showed most significant alterations. Further, hepatocellular carcinoma could be differentiated from cholangiocarcinoma based on the amounts of bile acids, phospholipids and cholesterol (Fig. 2) (*23*). Such a snapshot view of alerted bile homeostasis, is obtainable from a simple NMR approach and demonstrates the enormous opportunity to assess liver status, explore biomarkers for high risk diseases such as cancer, and improve the understanding of normal and abnormal cellular functions. Investigation of hepatobiliary diseases using human bile and NMR methods has the added advantage that results of such *in vitro* studies can be utilized for clinical applications using non-invasive *in vivo* magnetic resonance spectroscopy (*24*).

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Bile Acids Conjugation in Human Bile

In healthy individuals, bile acids perform a number of important functions including promoting cholesterol elimination by converting cholesterol to bile acids and transporting cholesterol from hepatocytes to intestine through micelles formation, transporting phospholipids through mixed micelles formation, fat absorption, and signaling bile acids synthesis through negative feedback regulation (*25*). In the small intestine, bile acids are thought to inhibit the growth of bacteria (*26*). Numerous studies link the bile acids to a number of hepatobiliary and intestinal diseases. As mentioned earlier, methodological developments in NMR have enabled single-step analysis of abundant and common glycine- and taurine- conjugated bile acids. Investigation of these conjugated bile acids in human bile employing high field NMR has shown that ratios between two glycine-conjugated bile acids and their taurine counterparts correlate positively as do ratios between a glycine-conjugated bile acid and its taurine counterpart (*27*). These insights into bile acids conjugation pattern in human bile between glycine and taurine promise useful clues to the mechanism of bile acids' biosynthesis, conjugation and enterohepatic circulation, and may improve our understanding of the role of individual conjugated bile acids in health and diseases.

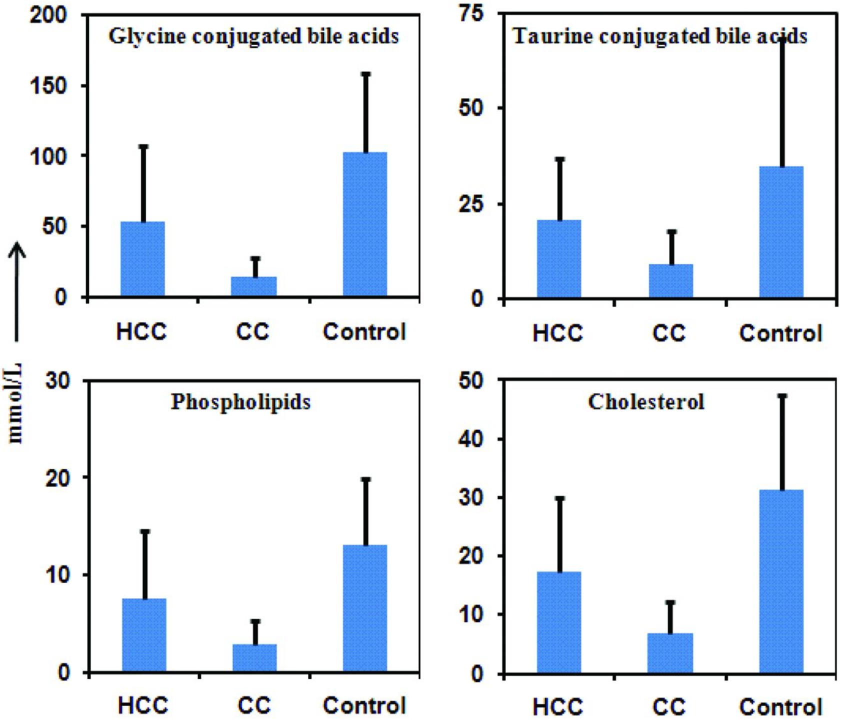

Figure 2. Distinguishing hepatocellular carcinoma (HCC) and cholangio-carcinoma (CC) from one another and from controls based on bile metabolite concentrations.

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Altered Bile Composition, Gallstones, and Gallbladder Cancer

Understanding the risk factors of gallbladder carcinogens in terms of bile composition and gallstones is an important step in the development of biomarkers for gallbladder cancer (GBC). Altered bile composition causes formation of gallstones (GS) in the gallbladder. GS disease is a common medical problem all over the world (*28*) and GBC is the most common malignancy (cancer) of the biliary tract. GS are suspected to be a contributing factor to the aetio-pathogenesis of GBC (*29*). GBC is frequently associated with GS (*30*) and the risk of GBC is 4 to 5 times higher in patients with GS than those without GS (*31*). At the same time, a very small number of patients with GS develop GBC. Several studies report the link between GS with GBC (*32–36*). While many studies report that patients with GBC are associated more frequently with large GS (*32–34*), a subsequent study contradicted such findings (*35*). Considering the importance of determining biochemical composition of GS under varied pathological conditions for understanding the aetio-pathogenesis of GBC, we recently analyzed bile metabolites in GS from patients with cancerous and non-cancerous gallbladder using NMR spectroscopy (*37*). Unlike gallstones from benign disease, those from GBC were cholesterol non-predominant mixed stones. Moreover, both calcium and magnesium were higher in GBC than in benign disease. Such differences in GS composition between malignant and benign gallbladder patients may provide useful clues to the aetio-pathogenesis of GBC and lead to identification of patients with GS, *in vivo*, who are at high risk of developing GBC and advocate prophylactic cholecystectomy to prevent GBC.

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Chapter 24

Influence of the Target Molecule on the ORAC Index

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The ORAC (Oxygen Radical Absorbance Capacity) assay has been widely employed to evaluate the antioxidant ability of pure compounds, foods and beverages. The method usually employs fluorescein and AAPH (2,2'-Azo-bis(2-amidinopropane) dihydrochloride) as target molecule (TM) and peroxyl radical source, respectively. According to a simple competition between TM and additives (antioxidants) for peroxyl radicals, the ORAC values (relative to a reference antioxidant), should be independent on the TM employed. In the present work we present ORAC values obtained employing different TM, such as fluorescein, alizarin red, pyrogallol red and pyranine. The results clearly show that the ORAC index of pure compounds and complex samples strongly depends on the TM used. Contradictory results on the antioxidant capacity of herbal and tea infusions can be obtained if fluorescein and pyrogallol red ORAC assays are compared. The dependence of the ORAC index on the TM emphasize the role of secondary radical reactions on the ORAC derived indexes.

1. Introduction

It has been demonstrated that the consumption of rich polyphenolic diets leads to a decreasing risk of cardiovascular diseases, hypertension, certain forms of cancer, type II diabetes, and other degenerative or age-related diseases

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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(*1–6*). These associations have been partially explained in terms of the ability of polyphenols to act as antioxidants through their reaction with Reactive Oxygen or Nitrogen Species (ROS, or RNS, respectively) (*1–6*).

Due to the complexity that supposes in vivo or ex-vivo experiments, different methodologies have been developed to estimate the in vitro antioxidant capacity of complex mixtures such as foods, beverages and human fluids (*7–12*). The methods, that involve different experimental conditions, evaluate: i) the consumption of stable free radicals induced by antioxidants (*13*, *14*); ii) the capacity of antioxidants to reduce cupric or ferric ions (*15*, *16*); iii) the ability of antioxidants to protect a target molecule exposed to a free radical source (*17*, *18*); iv) the capacity of antioxidants to inhibit lipoperoxidation processes (*19*), v) the antioxidant capacity of polyphenols in cell cultures (*20*), and the effect of antioxidants on the free radical steady state concentration (*21*).

2. ORAC (Oxygen Radical Absorbance Capacity) Assay

Among the above mentioned in vitro methodologies, ORAC is one of the most employed. This method estimates the ability of a particular sample to inhibit the bleaching of a target molecule (TM) induced by peroxyl radicals. Usually, Trolox or gallic acid are employed as reference antioxidants and 2,2'-Azo-bis(2-amidinopropane) dihydrochloride (AAPH) is employed as peroxyl radical source (*22*). To quantify the protection given by an additive it is estimated the area under the curve (AUC) of the kinetic profiles associated to the bleaching of the TM in the absence and presence of the tested sample (Figure 1). These data are compared to that of a reference antioxidant and the ORAC values estimated according to Equation 1 [eq 1], and Equation 2 [eq 2], for pure compounds and complex mixtures respectively:

$$ORAC = \frac{(AUC_{ad} - AUC^{0})}{(AUC_{Ra} - AUC^{0})} \frac{[Ra]}{[additive]} \qquad \textbf{[eq 1]}$$

$$ORAC = \frac{(AUC - AUC^{0})}{(AUC_{Ra} - AUC^{0})} f\,[Ra] \qquad \textbf{[eq 2]}$$

where:

AUC_{ad} = Area under curve in the presence of additives (pure antioxidants).

AUC = Area under curve in the presence of the tested complex mixture.

AUC^{o} = Area under curve of control (TM plus AAPH solution).

AUC_{Ra} = Area under curve in the presence of a reference antioxidant (usually Trolox or gallic acid).

f = Dilution factor, equal to the ratio between the total volume of the working solution (TM plus AAPH plus the sample aliquot) and the added sample volume.

[Ra] and [Additive] = Molar concentration of the reference antioxidant and additive, respectively.

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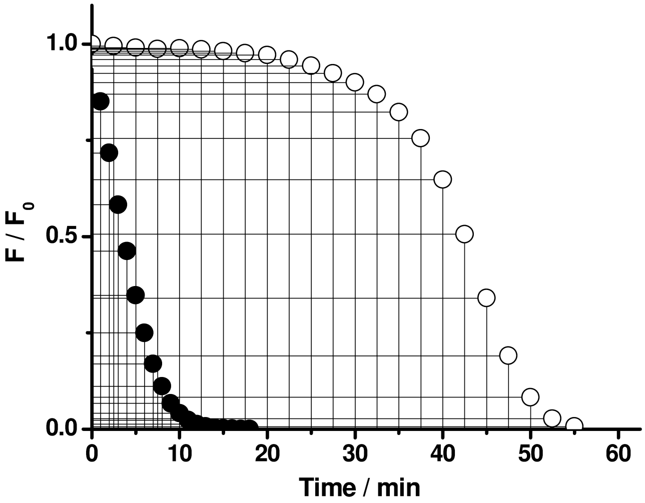

Figure 1. Kinetic profile of the TM consumption mediated by peroxyl radicals in the abscence (●) and presence of an antioxidant (○).

The higher AUC of the time-integrated bleaching of TM obtained in the presence of antioxidants (XH) can be explained by the minimal set of reactions presented in Scheme 1. This scheme represents a simple competition between TM and XH by peroxyl radicals. For simplicity self-reactions and cross-reactions of the radicals produced in steps [2] and [3], and the formation of alcoxyl radicals in reaction [4], are not included. According to this over simplified scheme, the AUC associated with reactions [1] to [4] will only depend on the difference of rate constant of process [2] and [3] and stoichiometric factors, following the reactions of TM and XH.

$$AAPH + O_2 \rightarrow 2ROO^{\bullet} + N_2 \qquad [1]$$

$$ROO^{\bullet} + TM \rightarrow bleaching \qquad [2]$$

$$ROO^{\bullet} + XH \rightarrow X^{\bullet} + ROOH \qquad [3]$$

$$2ROO^{\bullet} \rightarrow non \quad radicals \quad products \qquad [4]$$

Scheme 1

The ORAC method was firstly reported by Cao and co-workers employing R-phycoerythryn (PE) as TM (ORAC-PE) (*17*). ORAC-PE was used to estimate the antioxidant capacity of vitamin C, uric acid and human serum (*17*, *23–26*). However, it has been demonstrated that PE interacts with polyphenols and is a photounstable protein (*27*). Considering the latter, Ou and co-workers (*27*), reported an improved ORAC assay employing fluorescein (FL) as TM (ORAC-FL). FL does not interact with polyphenols, is photostable and it is considerable less expensive than PE. FL has been widely employed being, at present, the TM of choice.

In addition to the use of PE and FL, we have explore the use of other TMs, such as pyrogallol red (PGR) (*28*, *29*), pyranine (PY) (*30*, *31*), alizarin red (AR) (*32*), and c-phycocyanin (*33*, *34*) in ORAC-like methodologies. In this chapter it is discussed how the selection of the TM could affect the ORAC index. Results regarding the ORAC index of complex mixtures, employing PGR and FL as TM, are also presented.

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3. Influence of the Target Molecule (TM) on the ORAC Index of Pure Compounds

Figure 2 shows the Trolox inhibition on FL consumption-induced by peroxyl radicals. The presence of Trolox clearly inhibes the bleaching of FL. This protective effect is characterized by kinetic profiles with the presence of clear lag times. This behavior has been commonly obtained for different antioxidants (even for antioxidants of low reactivity) and would imply that FL is easily protected by additives almost irrespectively of their capacity to remove peroxyl radicals (*29*, *35*).

Similar results have been obtained when pyranine (PY) is used as TM (ORAC-PY). In Figure 3 it is shown the decay of the fluorescence of PY mediated by AAPH derived peroxyl radicals in the absence and presence of Trolox or gallic acid. As in Figure 2, the protection of PY is characterized by the presence of neat lag times in the kinetic profiles.

Alizarin red (AR) can also be used as TM (ORAC-AR) and its consumption can be easily followed by UV-visible spectroscopy. As in the case of FL and PY, the addition of antioxidants to a solution containing AR plus AAPH usually leads to kinetic profiles with lag times (Figure 4) (*32*).

Among the possible aspects that should be considered to explain lag times as those shown in Figures 2-4, are; i) a lower reactivity of the TM toward peroxyl radicals than the antioxidants, and ii) the presence of repair mechanisms (*30*, *35*). Nonetheless, independently of the origin of the lag time, its presence in the kinetic profiles implies that the antioxidant(s) is (are) totally consumed before the TM consumption. Thus, the lag times would be directly related to the stoichiometry of the reaction between antioxidant(s) and peroxyl radicals. Thereby, the AUC of kinetic profiles with lag times, and then, the related ORAC values, would be mainly associated to the stoichiometry of the antioxidant-peroxyl radicals reaction. Therefore, in many cases ORAC-FL, ORAC-PY and ORAC-AR values would be more influenced by the stoichiometry of the reaction (defined as the number of radicals that each additive molecule can remove) than by the reactivity of the additives toward peroxyl radicals.

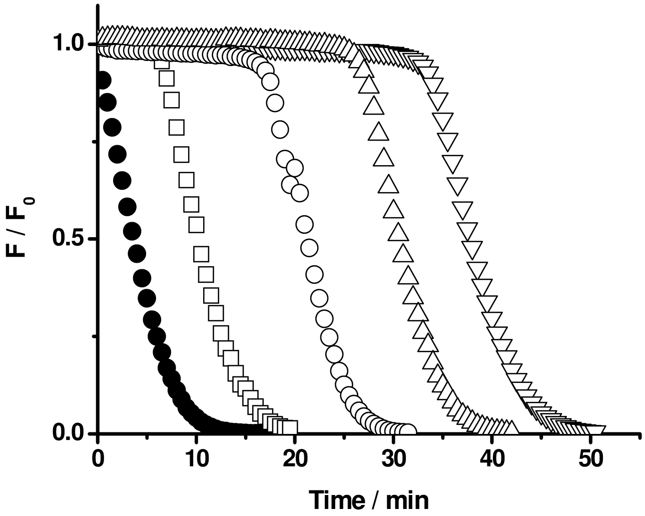

Figure 2. Time-course of the FL (70 nM) consumption induced by AAPH derived peroxyl radicals in the absence and presence of Trolox. Trolox concentrations: 1 μM (□); 5 μM (○); 7.5 μM (△); 10 μM (▽). Control experiment (●). [AAPH] = 10 mM. Data taken from ref. (29).

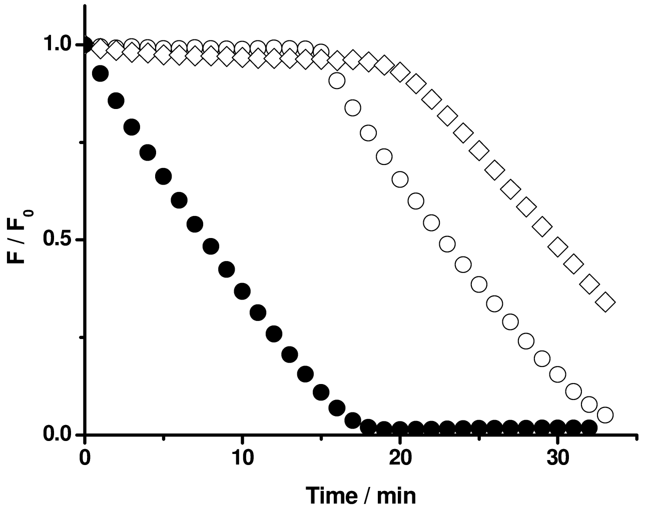

Figure 3. Time course of PY (5 μM) consumption induced by AAPH derived peroxyl radicals in the absence (●) and presence of Trolox (○) or gallic acid (◊). [Antioxidants] = 5 μM, [AAPH] = 10 mM. Phosphate buffer 10 mM, pH 7, 37°C.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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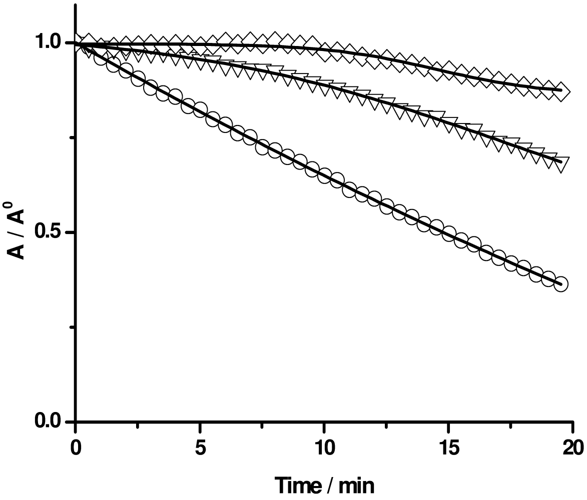

Figure 4. Consumption of AR (30 μM) elicited by AAPH (30 mM) derived peroxyl radicals in the presence of caffeic (4 μM, ▽) and sinapic acid (12 μM, ◊). Control experiment (in the absence of cinnamic acids, ○). The reaction was followed by visible spectroscopy at 520 nm. Data taken from ref. (32).

We have proposed that pyrogallol red (PGR) can be considered as TM in an ORAC-like methodology (ORAC-PGR) more sensitive to reactivity and less influenced by stoichiometric factors than FL or PY based methods (*28*, *29*). The time profiles associated to PGR consumption present clear differences with those depicted in Figures 2-4. PGR quickly reacts with AAPH-derived peroxyl radicals, and the reaction can easily be followed by the decrease of the absorbance of the sample measured at 540 nm. As it is shown in Figure 5, in parallel with the decrease at 540 nm (and at 280 nm), a new band at 380 nm is formed, a process that is characterized by the presence of isosbestic points at 325 and 435 nm.

In contrast to FL, PY and AR results, the protection of PGR afforded by Trolox is characterized by the absence of lag times (Figure 6). This behavior has been observed for different antioxidants, ascorbic acid being the only exception since its addition produces lag times in the kinetic profiles of the PGR consumption (*36*, *37*). The extent of the lag time can be considered as a measure of ascorbic acid concentration in tested complex samples.

The absence of lag times in the kinetic profiles of the consumption of PGR in the presence of antioxidants would imply that the AUC are mostly affected by the reactivity of the antioxidants toward peroxyl radicals. In spite of ORAC-PGR index would be more related to antioxidant reactivity than stoichiometric factors it can not be completely discarded the influence of secondary reactions. In fact, we have reported that the protection of PGR afforded by cinnamic acid derivatives is considerably smaller than those predicted from the reactivity of these antioxidants toward peroxyl radicals (*29*, *38*). This lack of protection could be interpreted in terms of secondary reactions, in which cinnamic acid derived radicals react with PGR (*39*).

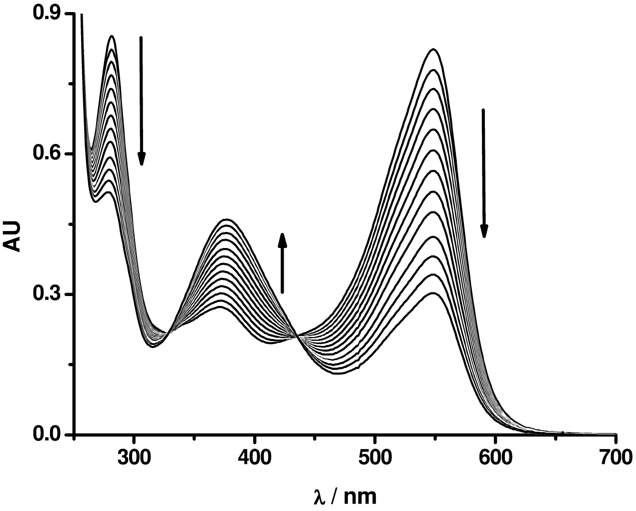

Figure 5. Bleaching of PGR (30 μM) induced by AAPH-derived peroxyl radicals. Curves represent the time course of the reaction between 0 and 50 minutes (28).

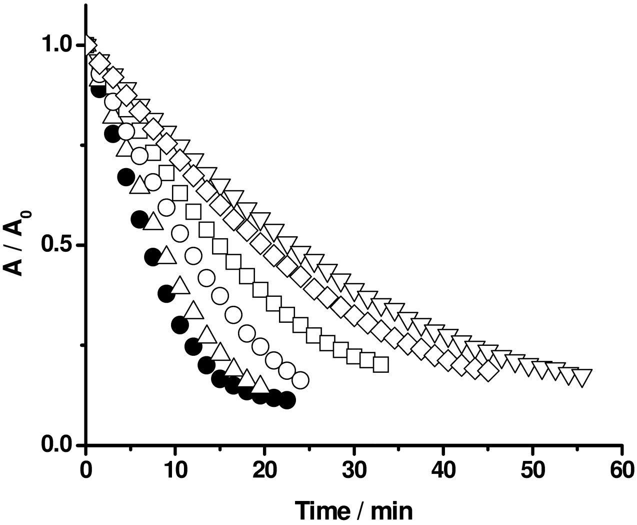

Figure 6. Time-course of the PGR consumption mediated by peroxyl radicals in the prescence of different Trolox concentrations. PGR (5 μM) was incubated with AAPH (10 mM) in the presence of Trolox at: 10 μM (△); 30 μM (○); 50 μM (□); 75 μM (◊); 100 μM (▽). Control experiment (in the abscence of Trolox, ●). Data taken from ref. (29).

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Table I shows ORAC values of pure compounds obtained employing FL, PY, PGR and AR as TM. As it is shown in this Table, the ORAC values are very dependent on the TM employed. For example, if the case of gallic acid is analyzed, ORAC-FL and ORAC-PY values of 1.2 and 1.4 were obtained, respectively. These values imply that the antioxidant capacity of gallic acid, estimated employing FL and PY as TM is close to that of Trolox. However, in contrast to ORAC-FL and ORAC-PY, the ORAC-PGR value of gallic acid is near to 11, implying that its antioxidant is much more efficient than Trolox.

Table I. ORAC values of pure compounds estimated using FL, PY, PGR and AR as TM. Data taken from ref. [a] (*29*), [b] (*27*), [c] (*40*), [d] (*31*), [e] (*32*)

Compound	*ORAC-FL*	*ORAC-PY*[d]	*ORAC-PGR*[a]	*ORAC-AR*[e]
Quercetin	10.7 ± 0.4[a] 7.28 ± 0.22[b]	6.4 ± 0.5	11.5 ± 0.4	---
Kaempferol	10.2 ± 0.3[a]	---	8.8 ± 0.7	---
Gallic acid	1.2 ± 0.03[a]	1.4 ± 0.1	11.1 ± 0.7	---
Caffeic acid	4.37 ± 0.24[a] 6.63 ± 0.24[c]	3.2 ± 0.3	≈ 0.2	2.1 ± 0.1
Ferulic acid	3.5 ± 0.1[a] 4.47± 0.1[c]	1.0 ± 0.1	≈ 0.1	0.2 ± 0.03
Sinapic acid	3.0 ± 0.1[a]	2.7 ± 0.3	≈ 0.4	1.9 ± 0.1
Trolox	1	1	1	1

The data depicted in Table I, clearly show that different TMs give different ORAC values, even for pure compounds. Furthermore, these results appear relevant if it is consider that all ORAC values are relative to the same reference antioxidant (Trolox). The latter can not be interpreted in terms of the simplified reactions depicted in Scheme 1 and emphasize the role of secondary reactions of TM or phenolic (additives) derived radicals (*35*, *39*).

The bleaching of a particular target molecule induced by peroxyl radicals involves, as a first step, an electron or hydrogen transfer in which a TM derived (TM$^{\bullet}$) radical is produced. If this radical is able to react with an antioxidant (XH) a repair mechanism would operative, according to:

$$TM^{\bullet} + XH \rightleftarrows X^{\bullet} + TMH \qquad [5]$$

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Thus, as is shown in reaction [5], the reduction of TM$^\bullet$ (mediated by XH) reduces the rate of TM bleaching. If this process is efficient, it could explain the presence of lag times in the kinetic profiles when FL and PY are used as target molecule (*30*, *35*). On the other hand, if XH reacts with peroxyl radicals, a XH secondary free radical (X$^\bullet$) is formed (reaction [3]). If X$^\bullet$ reacts with TM, a secondary damage of the target molecule would take place, according to:

$$TMH + X^\bullet \rightarrow XH + TM^\bullet \qquad [6]$$

This reaction would imply bleaching of TM induced by X$^\bullet$, as has been postulated in the protection of PGR elicited by cinnamic acids (*39*).

4. ORAC Values of Complex Mixtures Employing FL or PGR as Target Molecules (TM)

The ORAC-FL and ORAC-PGR methodologies have been applied to estimate the antioxidant capacity of complex mixtures such as herbal and tea infusions, and fruit berry extracts.

ORAC-FL and ORAC-PGR of Herbal and Tea Infusions

Herbal and tea infusions inhibited the consumption of FL through kinetic profiles characterized by the presence of lag times. However, the protection of PGR afforded by the same samples leads to changes in PGR initial consumption rate (without lag times). Figure 7 shows the dependence of ORAC values (ORAC-FL in plot A, and ORAC-PGR in plot B) with the total phenol content, evaluated by Folin assay, of herbal and tea infusions.

As it is shown in Figure 7, according to ORAC-FL method (Figure 7A), some herbal infusions presented higher antioxidant capacity than that of tea infusions with similar Folin values. However, all tea infusions show higher ORAC-PGR values than those of all herbal infusions (Figure 7B). Therefore, as in the case of pure compounds, ORAC values for different infusions depend upon the employed methodology. Thus, depending on the chosen TM opposite conclusions could be deduced.

Considering the characteristic of ORAC-FL and ORAC-PGR assays, we have proposed that the ratio between both indexes would indicate the quality of the antioxidants present in a particular sample. In Table II are presented the values of the ORAC-PGR/ORAC-FL ratio obtained for herbal and tea infusions.

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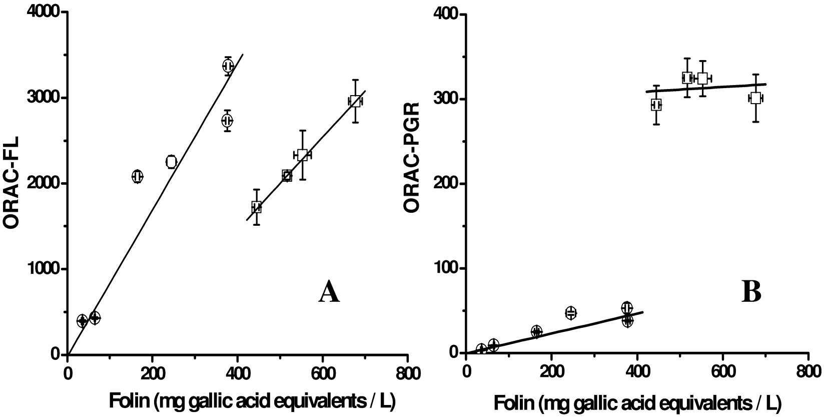

Figure 7. ORAC-index versus total phenolic content plots. Correlation between ORAC-FL (A) and ORAC-PGR (B) with total phenolic content of herbal (○) and tea (□) infusions. Data taken from ref. (41).

Table II. ORAC-FL/ORAC-PGR ratios of herbal and tea infusions. Data taken from refs. (*41*, *42*)

Infusion	*ORAC-PGR / ORAC-FL*	*Infusion*	*ORAC-PGR / ORAC-FL*
Chenopodium ambrosioides	0.008[a]	*Mentha piperita*	0.025
Buddleia globosa	0.011[a]	*Plantago major*	0.045
Erythroxylum coca	0.012	*Tilia spp*	0.055
Aloysia citriodora	0.021 (0.011[a])	*Black tea (1)*	0.10[a]
Matricaria chamomilla	0.021[a]	*Black tea (2)*	0.14[a]
Peumus boldus	0.019[a]	*Green tea*	0.15[a]
Rosa moschata	0.10	*White tea*	0.17[a]
Haplopappus baylahuen	0.020[a]		

As can be seen in Table II, the ORAC-PGR/ORAC-FL ratio depends on the tested sample. For example, the ratio of *Peumus boldus* is close to 8 times smaller than that of green tea. This would indicate that the antioxidants present in green tea are more efficient that the antioxidants present in the *Peumus boldus* infusion. In this context, a similar approach, employing PY and PGR as TM, has been proposed by Niki and co-workers to estimate the antioxidant capacity of beverages and foods (*43–45*).

ORAC-FL and ORAC-PGR of Berry Extracts

Blue and black berry extracts protect PGR from its consumption mediated by AAPH derived peroxyl radicals. This protection is characterized by the absence of lag times. On the other hand, kinetic profiles related to the protection of PGR afforded by raspberry extract (Figure 8) are characterized by the presence of clear lag times. Considering previous studies that indicated that ascorbic acid generates lag times in the protection of PGR (*28*), experiments in the presence of ascorbate oxidase were carried out. The preincubation of raspberry extract with this enzyme completely eliminated the lag time, without modifying the slope observed after the lag time (*36*). The raspberry extract protected FL throughout kinetic profiles with neat lag time, but the addition of ascorbate oxidase did not modify these kinetic profiles. The latter implies a minimal contribution of ascorbic acid on the AUC when FL is employed as TM (*36*).

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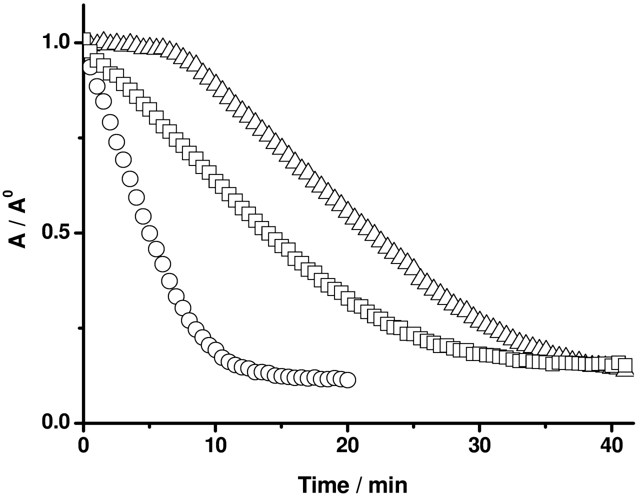

Figure 8. Effect of ascorbate oxidase on the kinetic profiles of the PGR consumption in the presence of raspberry extract. Previously to PGR (5 μM) and AAPH (10 mM) addition, the raspberry solution (33 μL of extract / mL in phosphate buffer, 75mM, pH 7.4) was incubated with ascorbate oxidase (0.09 U/mL) during 40 minutes. Solution of PGR in the presence of both, raspberry extract (33 μL / mL) and AAPH (10 mM) = (△). Pre-incubated solution with ascorbate oxidase (□). Control experiment (○). Data taken from ref. (36).

These results show that in rich ascorbic acid mixtures, the use of PGR as TM allows an estimation, throughout a simple methodology, of the ascorbic acid concentration (from the lag time) and the ORAC-PGR value of the sample. This allows to estimate the contribution of ascorbic acid to the antioxidant capacity of a particular complex mixture. In raspberry extracts an ORAC-PGR value of 1453 micromolar Trolox equivalents was estimated. However, without the contribution of ascorbic acid, an ORAC-PGR value of 489 was determined. The presence of ascorbate oxidase enzyme did not modify the AUC of the kinetic profiles of FL consumption. This imply that the ORAC-FL value of raspberry extract does not reflect the antioxidant capacity given by ascorbic acid. In fact, the ORAC-FL value of raspberry extract was 2870 micromolar Trolox equivalents in the absence and presence of ascorbate oxidase (*36*).

5. Conclusions

ORAC values of pure compounds and complex samples strongly depend on the target molecule. This dependence emphasize the role of secondary radical reactions on the ORAC derived indexes. These reactions could partially explain contradictory ORAC values obtained for berry fruits and herbal and tea infusions employing fluorescein or pyrogallol red as target molecules..

Acknowledgments

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Chapter 25

Inhibition of Intestinal α-Glucosidases and Anti-Postprandial Hyperglycemic Effect of Grape Seed Extract

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Because intestinal α-glucosidase plays a key role in carbohydrate digestion, its inhibition provides a therapeutic option for diabetes by suppressing postprandial blood glucose. We recently identified a grape seed extract (GSE) significantly inhibiting α-glucosidases. The inhibitory activity of GSE on yeast α-glucosidase was significantly stronger than that of acarbose. GSE also inhibited rat α-glucosidases *in vitro* in a dose- and time-dependent manner. The potential anti-diabetic effect of GSE was further evaluated in an animal model. Male 6 week-old C57BLK/6NCr mice were treated by streptozocin to induce diabetes. The results showed the oral intake of GSE (400mg/kg, body weight) suppressed postprandial blood glucose in STZ-induced diabetic mice. Oral administration of GSE reduced postprandial blood glucose in the diabetic mice by 11.5% and 16.6% at 30 and 60 min after the starch meal. Overall, GSE intake significantly reduced the incremental $AUC_{0\text{-}120min}$ (area under postprandial glycemic curve) by 27.3% as compared to the control. Our results strongly suggest the potential of developing GSE, as a novel inhibitor of α-glucosidases, for diabetes prevention and treatment.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Introduction

Diabetes has been one of the major public health problems in the United States. Diabetes is at least in part related to the amount of carbohydrates in the diet. The digestion of dietary carbohydrates such as starch primarily occurs in the small intestine by α-amylase to yield both linear maltose and branched isomaltose oligosaccharides, neither of which can be absorbed into the bloodstream without further hydrolysis by α-glucosidases to release glucose (*1*). Therefore, intestinal α-glucosidase is critical in carbohydrate digestion and glucose release, and its inhibition provides an important anti-diabetic option by reducing postprandial hyperglycemia.

Postprandial hyperglycemia is an early symptom of type 2 diabetes (*2*), which occurs when pancreatic β cells fail to secrete a sufficient amount of insulin (*3*). Postprandial hyperglycemia induces glucose toxicity and further deteriorates β cell function (*4*, *5*). Treatment of postprandial hyperglycemia has been proven to improve overall glycemic control (*6–9*). On the other hand, inhibition of α-glucosidases has been demonstrated to be effective in both preventing and treating diabetes through improvement of postprandial hyperglycemia (*1*, *10–14*). Commercial inhibitors, in particular acarbose, have been used for diabetes treatment. However, acarbose has been associated with significant adverse gastrointestinal (GI) side effects which is partly attributable to its non-specific inhibition of α-amylase, causing excessive accumulation of undigested carbohydrates in the large intestine (*8*, *15*). Therefore, specific α-glucosidase inhibitors may provide a novel antidiabetic effect but with fewer GI side effects than currently available inhibitors.

We recently found that a grape seed extract (GSE) significantly inhibits the activity of α-glucosidase and the inhibition appears to be selective because GSE does not inhibit structure-comparable α-amylase, suggesting that GSE may be a specific α-glucosidase inhibitor. This study aimed to determine time- and dose-responses of GSE on α-glucosidases and its inhibition mode, and to further assess whether acute intake of GSE can suppress postprandial blood glucose in diabetic animals.

Materials and Methods

Materials

Yeast type I α-glucosidase (EC 3.2.1.20, G5003), rat intestinal acetone powder (N1377-5G), *p*-nitrophenyl α-D-glucopyranoside (pNPG), acarbose, porcine pancreatic α-amylase, type VI-B (A3176), porcine pancreatic lipase, Type II (L3216), and streptozotocin (STZ) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The organic solvents used in this study were HPLC grade (Fisher Scientific Co.).

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GSE Preparation

Grape seeds were obtained from a local Virginia vineyard (Blackstone, VA, USA). The seeds were then ground to fine powder by a Thomas Wiley mini-mill (Swedesboro, NJ). The samples were extracted with 80% ethanol at 1:10 ratio (m/v) under overnight shaking. The extracts were filtered through Whatman No. 4 filter paper to remove unwanted residues. After evaporating off the organic solvent, the filtrates were frozen and lyophilized to obtain GSE.

Yeast and Mammalian α-Glucosidase Inhibition Assays

Both the yeast and mammalian α-glucosidase activity was assayed using the substrate pNPG, which is hydrolyzed by α-glucosidase to release the product p-nitrophenol, a color agent that can be monitored at 405 nm (*16*). The yeast α-glucosidase (EC 3.2.1.20) is categorized as type I α-glucosidase and the inhibition assay was conducted according to the previous assays with slight modification (*16, 17*). In brief, 80 µl of each sample solution (1mg/ml) was mixed with 20 µl of the enzyme solution (1 U/ml) and incubated in a 96-well plate at 37ºC for 3 min. After incubation, 100 µl of 4 mM pNPG solution in 0.1 M phosphate buffer (pH 6.8) was added and the reaction was conducted at 37ºC. The release of p-nitrophenol was monitored at 405 nm every minute for 75 min spectrophotometrically (Victor, PerkinElmer, USA). The α-glucosidase activity was determined by measuring area under the curve (0-75 min) for each sample and compared with that of the control where GSE was replaced by the dissolving solvent.

The mammalian α-glucosidases were prepared from 1g of rat intestinal acetone powder suspended in 20 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM EDTA at ambient temperature. The suspension was sonicated for 15 min and after vigorous stirring for 1 h, the suspension was centrifuged (at 12000xg for 15 minutes). The supernatant was dialyzed against 0.01 M potassium phosphate buffer (pH 7.0) for 24 hours. The activity of rat α-glucosidase extract was verified using pNPG as the substrate by comparing with the pure yeast α-glucosidase. The assays were conducted as described above. Acarbose was used as a positive control.

Pancreatic α-Amylase Inhibition Assay

The α-amylase inhibitory activity was determined using Type VI-B porcine pancreatic α-amylase (*18*). In brief, 500 µl of GSE dilutions and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α-amylase solution (0.5 mg/ml) were incubated at 25 °C for 10 min. After preincubation, 500 µl of a 0.5% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube at timed intervals. The reaction was stopped with 1.0 ml of dinitrosalicylic acid color reagent. The test tubes were then incubated at 90 °C in a water bath for 10 min and cooled to room temperature. The reaction mixture was then diluted 1:15 with distilled water, and absorbance was measured

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
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at 540 nm with the PerkinElmer plate reader. The readings were compared with the controls, containing buffer instead of sample extract. The results were expressed as percent α-amylase inhibition.

Pancreatic Lipase Inhibition Assay

The inhibition of pancreatic lipase by GSE was determined by measuring the amount of 4-methylumbelliferone product released by lipase spectrofluorometrically using the plate reader (*18*). Pancreatic lipase (Type II, from porcine pancreas) and 4-methylumbelliferyl oleate (4-MU oleate) served as the reaction enzyme and substrate, respectively. The reaction mixture was prepared with 25 μl of the GSE dilutions and 25 μl of 16.7 U/ml lipase in Tris-HCl, pH 8.0 buffer solution. The reaction was initiated by adding 50 μl of 0.1M 4-MU oleate in Tris-HCl, pH 8.0 buffer solution. After incubation at 37°C for 30 min, the rate of release of the 4-methylumbelliferone product was measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm. Commercial lipase inhibitor, Orlistat, was used as the positive control. The readings were compared with the controls, containing buffer instead of sample extract. The results were expressed as percent α-lipase inhibition.

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Time and Dose-Responses of GSE on Yeast α-Glucosidases

The enzyme reactions were performed on various GSE concentrations (2.9-285.7 μg/ml) within 75 min. The assays were conducted under the same conditions as described above and the release of p-nitrophenol was monitored every 5 min.

IC_{50} of GSE against Mammalian Intestinal α-Glucosidases

To determine IC_{50} of GSE on rat intestinal α-glucosidases, we conducted the enzymatic reactions with various GSE concentrations (0.2-1mg/ml) incubated with pNPG ranging from 0.4-2 mM.

Animal Experiments

Animals

Male 6-week old mice (C57BLKS/6NCr, National Cancer Institute, Frederick, MD, USA) were housed in groups of four mice per cage and maintained on a 12-hour light-dark cycle at 20 °C to 22 °C. Mice were acclimatized for a 2-week period before starting the experiment and had *ad libitum* access to food and water. Mice were maintained on rodent feed (Harlan Tekland Gobal Diets 2018 rodent diet containing 60% of calories from carbohydrate, 23% of calories from protein, and 17% of calories from fat; digestible energy of 3.4 Kcal/g, Madison WI, USA) for the duration of the experiment. Animal husbandry, care, and experimental procedures were conducted in compliance with the "Principles of Laboratory Animal Care" NIH guidelines, as approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech.

STZ Induction of Diabetes in Mice

Diabetes was induced by intraperitoneal injection of STZ dissolved in 10 mM sodium citrate buffer (pH 4.5) at a dose of 50 mg/kg body weight (bw). The STZ was dissolved in ice-cold citrate buffer protected from light and injected immediately to avoid STZ degradation. Five to seven days after STZ injection, mice with non-fasting blood glucose ≥250 mg/dl were considered to have diabetes (*19*).

Oral GSE Treatment and Starch Challenge

The experiment was designed to determine the effect of acute intake of GSE on postprandial glycemic response in STZ-induced diabetic mice following a potato starch challenge. Mice were fasted for 14 hours in freshly cleaned cages with free access to water before the experiment (4/group). Mice in the control group were given 0.2 mL of water by oral gavage. The treatment group were administered 0.2 mL of GSE suspension (400 mg/kg bw) by oral gavage immediately after vortexing the suspension. After approximately 30 minutes post water or GSE administration, 0.2 mL of potato starch suspension (2 g/kg bw) was administered to each mouse by gavage. Approximately 5 µL of whole blood samples were collected from the tail vein of each mouse. The blood samples were acquired at 0, 30, 60, and 120 minutes after the oral starch challenge. Blood glucose levels were measured with a blood glucometer and accompanying test strips (ACCU-CHEK Meter®, Roche Diagnostics, Kalamazoo, MI). The area under the glucose tolerance curve ($AUC_{0-120\,min}$) was calculated using a trapezoidal method (*20*). The total antihyperglycemic response ($AUC_{0-120\,min}$) was expressed as mean ± standard deviation.

Statistical Analysis

The statistical significance comparing data between groups was assessed by compared by a two-sample *t*-test or one-way analysis of variance (ANOVA) followed by Duncan's multiple range post-hoc tests. Statistical analysis was performed using SPSS (Windows, Version Rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Statistical significance was declared when $P < 0.05$.

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Results

Time and Dose-Responses of GSE against Yeast α-Glucosidase

Figure 1A shows time-responses of GSE at different concentrations against yeast α-glucosidase. The inhibition of yeast α-glucosidase activity by GSE was sustainable during 75 min of the reaction period. The significant inhibition was seen when GSE concentration was as low as 1.4 µg/ml. A dose-dependent inhibition of yeast α-glucosidase by GSE was also observed (Figure 1B). The inhibitory activity of GSE was compared with acarbose. GSE at 1.4 µg/ml exerted significantly stronger inhibition than acarbose at 285.7 µg/ml. GSE inhibited more than 90% of yeast α-glucosidase activity at a concentration of 28.6 µg/ml or higher. We further determined the IC_{50} of GSE against yeast α-glucosidase which was 1.5 µg/ml. Furthermore, we examined whether GSE also inhibits other digestive enzymes including pancreatic α-amylase and lipase. However, GSE at comparable or even higher concentrations showed no significant inhibition on both enzymes with GSE concentrations up to 1 mg/ml.

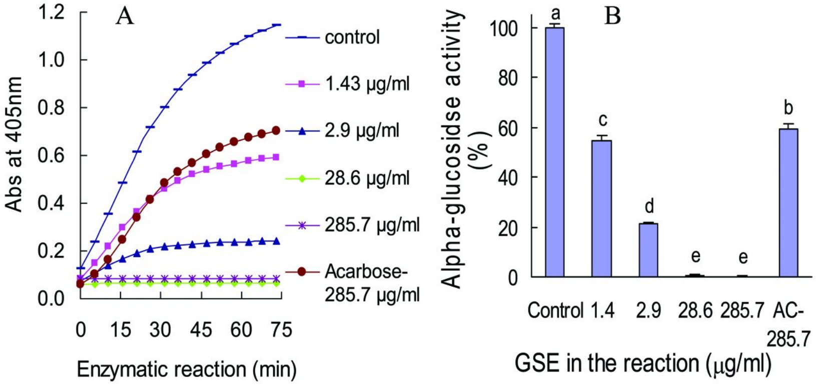

Figure 1. Kinetics of yeast α-glucosidase inhibition by GSE at different concentrations (A); Dose-dependent inhibition of GSE on yeast α-glucosidase (B). Enzyme activity was determined by measuring p-nitrophenol released from pNPG at 405 nm. Acarbose is the positive control and denoted as AC. Bars with the different letters are significantly different ($p < 0.05$).

Dose-Dependent Inhibition of Mammalian Intestinal α-Glucosidase by GSE

GSE also significantly inhibited the activity of rat intestinal α-glucosidases. As shown in Figure 2, the enzymatic inhibition was highly dose-dependent. GSE inhibited the activity of rat α-glucosidases by 25.4%, 69.8%, and 78.6% at the doses of 0.4, 1, and 2 mg/ml, respectively. For comparison, acarbose at 0.08 mg/ml showed 47.2% inhibition of rat α-glucosidases. The IC_{50} of GSE was determined to be 0.73 mg/ml on rat α-glucosidases.

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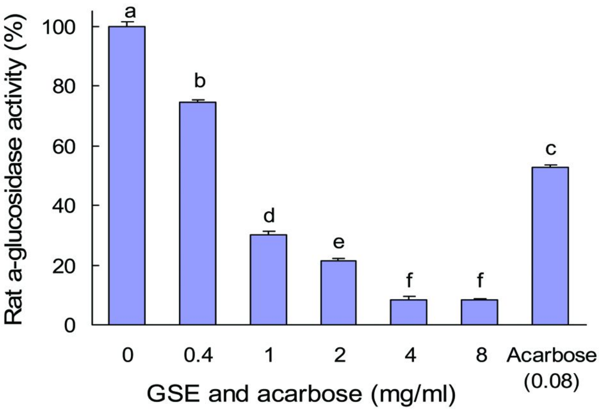

Figure 2. Dose-dependent Inhibition of GSE on rat α-glucosidases. Rat intestinal α-glucosidase activity was determined by measuring p-nitrophenol released from pNPG at 405 nm. The reaction was conducted at 37ºC for 75 min. Bars marked by different letters are significantly different (p < 0.05).

Inhibition of Postprandial Blood Glucose by the Acute Intake of GSE in STZ-Treated Mice

Figure 3 shows the effect of GSE on postprandial blood glucose in STZ-induced mice after oral loads of potato starch (Fig 3A & 3B). Oral administration of GSE (400mg/kg, bw) reduced postprandial blood glucose by 11.5% and 16.6% at 30 and 60 min after the starch meal, respectively (Fig 3A, $P > 0.05$). Overall, GSE intake reduced the incremental $AUC_{0\text{-}120min}$ (area under postprandial glycemic curve) by 27.3% as compared to the control ($P < 0.05$).

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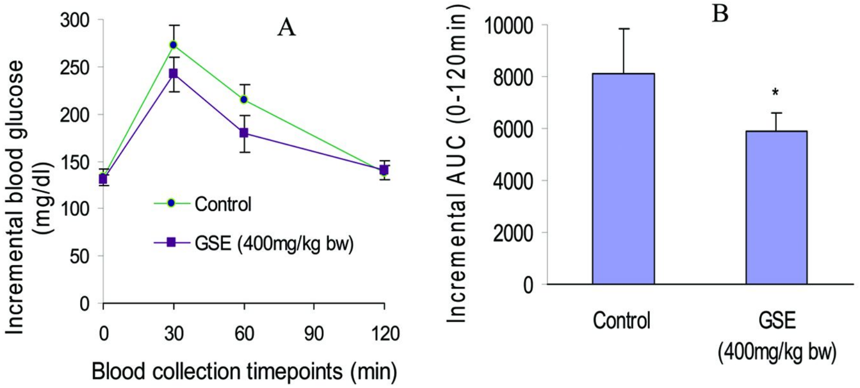

Figure 3. Postprandial glycemic response after acute intake ofGSE in STZ-treated mice after a starch meal. The glycemic response curve in diabetic mice after starch challenge (A).The incremental $AUC_{0\text{-}120min}$ in diabetic mice after starch administration (B); The fasted diabetic mice were administered with 100µl of either vehicle or GSE suspension (400mg/kg, bw) by gavage. After 30 min, 100µl of potato starch suspension (2g/kg, bw) was administered and blood was collected from tail vein at 0, 30, 60, and 120 min to determine glucose levels., P<0.05 vs. control.*

Discussion

The primary goal of this study was to identify novel bioactive food components with specific inhibitory activity against α-glucosidase but not α-amylase. GSE was identified in our preliminary screening experiments using yeast α-glucosidase because the enzyme is readily available in pure form and has been used by others for nutraceutical and medicinal investigations (*16*, *17*, *21*, *22*). In the subsequent experiments, GSE demonstrated significantly stronger inhibitory activity against yeast α-glucosidase than acarbose. GSE at the concentration as low as 1.4 µg/ml inhibited 45.4% of the enzyme activity compared with 40.7% inhibition by 285.7 µg/ml of acarbose. We further examined its effects on other digestive enzymes such as pancreatic α-amylase and lipase. Alpha-amylase (E.C. 3.2.1.1) and α-glucosidase (EC 3.2.1.20) belong to the glycoside hydrolase family 13 and both enzymes share a common reaction mechanism and several short conserved sequences (*23*). Non-specific inhibitors likely inhibit both enzymes due to the structure similarities of the enzymes. However, GSE showed no significant inhibition on α-amylase even at concentrations up to 1mg/ml. Moreover, no inhibition of pancreatic lipase was detected by GSE (data not shown). Collectively, these data suggest GSE could be a specific inhibitor of α-glucosidases and the underlying mechanism may differ from that of acarbose, which inhibits both α-amylase and α-glucosidase.

We further evaluated the inhibitory effect of GSE on α-glucosidases prepared from a mammalian source which is more biologically relevant. The enzyme was extracted and purified from rat intestinal powder. The result showed that GSE also significantly inhibited the activity of rat α-glucosidases and the inhibition was highly dose-dependent. The IC_{50} of GSE on rat α-glucosidases was 0.73 mg/ml. It appears that GSE was much more effective inhibiting yeast α-glucosidase than mammalian α-glucosidases. The inhibitory activity of GSE on mammalian α-glucosidases was comparable to other reported natural inhibitors such as oolong tea extract (IC_{50}=1.34 mg/ml) and green tea extract (IC_{50}=0.735 mg/ml) (*22*). Dietary supplementation of green tea extract at 180-300 mg/kg has shown significant anti-hyperglycemic effects without affecting the levels of insulin in diabetic mice (*24*) and rats (*22*, *25*). Green tea is rich in polyphenols particularly catechins. However, we found that catechin does not inhibit rat intestinal α-glucosidases. GSE contains a number of proanthocyanidins. Some proanthocyanidins have been shown to inhibit yeast α-glucosidase (*26*). It is unclear whether these compounds are responsible GSE-induced inhibition of α-glucosidases. We are currently working on the isolation and identification of the active compounds in GSE.

The strong *in vitro* inhibitory activity of GSE on rat intestinal α-glucosidase has prompted us to examine whether GSE can also inhibit α-glucosidase *in vivo*, thereby slowing starch digestion and subsequently reducing postprandial blood glucose. This can be done on diabetic mice. Male 6-week old C57BLK/6NCr mice were therefore induced to diabetes by STZ injection and were used to assess the effects of oral GSE intake on postprandial blood glucose. The acute intake of GSE (400 mg/kg, bw) significantly reduced postprandial blood glucose by 27.3% (the incremental $AUC_{0\text{-}120min}$) in diabetic mice after a starch meal, though the significant differences were not shown in individual time points. We speculate that the suppression of postprandial blood glucose by GSE intake might be related to its inhibition of intestinal α-glucosidase in mice. However, we are unable to directly determine how intestinal α-glucosidase activity is affected *in vivo* after ingestion of GSE partly because the enzymes are immobilized in the small intestine. Collectively, the animal results suggest that GSE may have the unique property of suppressing the absorption of glucose *in vivo* through a potential mechanism involving the inhibition of small intestinal α-glucosidases.

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Chapter 26

Fruit and Vegetable Polyphenol Consumption Decreases Blood Pressure

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Hypertension is one of the main cardiovascular risk factors, modifiable by diet. On the other hand, an inverse association between risk for cardiovascular disease and consumption of polyphenol-rich foods has been found in several epidemiologic studies. The aim of the current study was to evaluate the usefulness of total polyphenols excreted in urine as a new biomarker of total polyphenols intake and to correlate it with blood pressure (BP) and the prevalence of hypertension in a large cohort of high-risk subjects included in the PREDIMED trial. Participants in the highest quartile of urinary total polyphenols excreted had a 36% reduced prevalence of hypertension compared to those in the lowest quartile. In addition, systolic and diastolic BP were inversely associated with urinary total polyphenols excreted after adjustment for potential confounders (P <0.05) in an elderly Mediterranean population at high cardiovascular risk. We concluded that high consumption of polyphenol-rich foods reduces the prevalence of hypertension.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Essential hypertension, defined as systolic blood pressure (BP) greater than 140 mmHg or diastolic BP greater than 90 mmHg, is the major cause of cardiovascular morbidity, as well as the major modifiable cause of death in both economically emerging and developed countries (*1*). In population studies, a reduction in the entire distribution of BP by 5 mmHg has been hypothesised to produce a 40% decrease in the incidence of stroke and a 20–25% reduction in CHD (*2–4*).

Lifestyle changes may help to prevent hypertension. These changes include the maintenance of a body mass index (BMI) between 18.5 and 24.9 Kg/m^2, reduction of sodium intake to less than 2,300 mg a day, which is about 1 teaspoon of salt, and to perform exercise that raises heart rate at least 2½ hours a week. Other measures are to follow-up a healthy diet and limit alcoholic beverages to 2 drinks a day for men and 1 drink a day for women. In fact, the Mediterranean (*5*) and the DASH (Dietary-Approaches-to-Stop-Hypertension) diets (*6*) have also demonstrated to reduce high BP. Similarly, other clinical trials have observed that following a diet rich in fruits, vegetables and cocoa also decreases BP (*6–10*). However, up to now, clinical trials focused on the relationship between phenolic compounds from fruits and vegetables (F&V) and BP have reported conflicting results, mainly because the food studied had very different phenolic profile and the effect of microbiota was not well established.

There is evidence suggesting that the high consumption of F&V lowers BP and may protect against CVD and stroke (*11–15*). F&V contains high amounts of polyphenols, micronutrients that have exhibited a broad spectrum of biological activities (*16*) for human health including anti-hypertensive properties (*17*). Table I summarizes the results of different intervention clinical trials with polyphenol-rich foods on BP (*18–33*). It is difficult to draw conclusions since some results are contradictory and many variables may affect the results observed, such as subject characteristics (healthy, hypertensive or with other cardiovascular risk factors), the amount and class of polyphenols given, food matrix, and duration of the study. Thus, for instance, to lower blood pressure is more difficult in normotensive than in hypertensive subjects. Another example of variability is the different results obtained in various studies using pomegranate juice. A consistent 5% reduction in systolic BP was reported when Aviram *et al.* (*18*) gave 50 mL/d pomegranate juice to 10 hypertensive individuals for 2 weeks, whereas 21% reduction in systolic BP was observed when the same volume of juice was given to a larger group of participants with asymptomatic severe carotid artery stenosis for a year (*19*). By contrasts, Sumner and colleagues (*21*) reported a reduction in stress-induced ischaemia, but no effect on BP after 240 mL/d of pomegranate juice, even though a larger volume than Aviram's was consumed for a longer length of time (90 d) in a much larger group of participants (n = 45) with ischaemic coronary disease (*20*). The explanation for these discrepant results is unclear and counterintuitive. A possible reason is that juices used in the studies were derived from different sources and thus had different polyphenolic content. Sumner's group used a commercial pomegranate juice, which suffers more technologic processing that may affect the polyphenolic composition, while Aviram's group produced an in-house concentrated form of pomegranate juice, which was chemically analysed.

The differences in the health status of the participants in the studies and the length of dietary intervention may also explain part of these discrepancies. Thus, available data is weak since it include few and small studies. Therefore, long-term clinical or epidemiological trials are needed to definitively clarify the benefits deriving from long-term consumption of polyphenol-rich foods or a polyphenol-rich diet pattern.

In epidemiological trials, biomarkers of the intake of some nutrients are more precise and provide better objective measures than data obtained from food frequency questionnaires (FFQ). The development of biomarkers, measured in blood or urine, is essential for making accurate estimates of polyphenol intake. However, the relationship between dietary intake and nutritional biomarkers has been often highly complex (*34*).

The major and still unresolved drawback in evaluating polyphenol bioavailability is the fact that after strict dietary monitoring (i.e., diets free of those phenolic compounds of interest), and following hours of fasting, it remains impossible to eliminate all phenolic compounds in biological fluids (*35*). Therefore, a basal concentration of phenolics will be found in the urine, even avoiding polyphenol-containing food intake for some days.

Water accounts for about 95% of the total volume of urine and the remaining 5% consisting of solutes derived from cellular metabolism and outside sources. A wide range of water-soluble compounds, including mineral salts, vitamins, amino acids, enzymes, hormones, antigens, fatty acids, nucleosides, immunoglobulins, pigments, uric acid, urea, hippuric acid, etc, are believed to be present in urine normally, although other substances like proteins, glucose, erythrocytes, and ketones bodies can also be found when the body's processes are not operating efficiently (*36*). Thus, the Folin-Ciocalteu (F-C) assay could prove a poor method for determining the total phenolic concentration in urine, due to the above-mentioned interfering elements. However, we reported that the application of a Solid Phase Extraction (SPE) procedure to urine samples can remove such reductant water-soluble compounds. Following this with the Singleton and Rossi F-C assay (*37*) with certain modifications, provides an effective technique to measure total phenolics compounds excreted in urine (*38*). SPE with 96-well plate cartridges (Oasis® MAX) was performed in the urine samples to avoid any interference with F-C reagent. For all the spot urine samples, total polyphenols excreted (TPE) was analyzed as described by Medina-Remón A. *et al* (*38*) and expressed as mg gallic acid equivalent (GAE)/g of creatinine. To ensure that there are not interfering reductant substances, an evaluation of the main reductant substances from urine and the major drugs consumed in Europe was performed (see Table II). Before SPE cleaning up procedure, some interfering substances at the level that may be present in urine react with the F-C reagent (Fe(II), Vitamin C, adrenaline, noradrenaline and dopamine; however after the SPE clean-up any of them react with F-C. From the drugs studied, only paracetamol can give interference, after the cleaning process, on TPE results and therefore paracetamol intake should be registered in all questionnaires and evaluations.

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Table I. Effects of food polyphenols on blood pressure in human intervention studies

References	*Type of study*	*No. Ind.* [†]	*Subjects' characteristics*	*Age range years*	*Substance given*	*Main polyphenols* [‡]	*Dose/d (amount of polyphenols)*	*Duration*	*Biomarkers*	*Main changes on BP*[¥]
Aviram & Dornfeld (2001) (*18*)	Chronic, single arm, no control	10 (7m,3f)	Hypertensives	62-77	Pomegranate juice	Tannins,anthocyanins	50 mL (1.5 mmol)	14 days	SBP serum ACE	↓5% ↓36%
Reshef et al. (2005) (*19*)	Chronic, controlled parallel	12 (8m, 4f)	Stage I Hypertensives	42-62	Sweetie fruit (hybrid between grapefruit and pummelo)	Flavonoids	0.5 L (889 mg/L)	5 weeks	SBP DBP	?
Aviram et al. (2004) (*20*)	Chronic, controlled parallel	19 (14m,5f)	Patients with asymptomatic severe carotid artery stenosis	65-75	Pomegranate juice	Tannins,anthocyanins	50 mL (2484 mg/L)	1-3 years	SBP (after 1 y) SBP (after 3 y) DBP	↓ 12% No further reduction ↔
Sumner et al. (2005) (*21*)	Chronic, controlled parallel	45 (40m,5f)	Patients with ischaemic coronary disease and myocardial Ischaemia	58-80	Pomegranate juice	Tannins,anthocyanins	240 mL	90 days	BP	↔

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References	*Type of study*	*No. Ind. †*	*Subjects' characteristics*	*Age range years*	*Substance given*	*Main polyphenols ‡*	*Dose/d (amount of polyphenols)*	*Duration*	*Biomarkers*	*Main changes on BP¥*
Ruel et al. (2005) (*22*)	Chronic, single arm, no control	21 (all men)	Healthy	30-46	Cranberry juice	Flavonoids, phenolic acids	7 mL/Kg body wt.	14 days	SBP	↓ 2% (not statistically significant)
Taubert et al. (2003) (*23*)	Acute, controlled crossover	13 (6m,7f)	Healthy but with stage 1 mild isolated systolic hypertension	55-64	Dark chocolate	Flavonoids	100 g (500 mg)	14 days	SBP DBP	↓ 5.1 mmHg ↓ 1.8 mmHg
Gorinstein et al.(2006) (*24*)	Chronic, controlled parallel	57	hyperlipidaemics after coronary bypass surgery	39-72	Blond or red grapefruit	Flavonoids, anthocyanins	One fruit (20 mg/100 g fresh wt.)	30 days	SBP DBP	↔ ↔
Naruszewicz et al.(2007) (*25*)	Chronic, controlled parallel	44 (33m,11f)	myocardial infarction survivors on statins for 6 months	57-75	Chokeberry flavonoid extract	Anthocyanins, procyanidins	3 × 85 mg	42 days	DBP SBP serum ACE	↓ 7.2 mmHg ↓11 mmHg ↓33.3%
Taubert et al. (2007) (*26*)	Chronic, controlled parallel	44 (20m,24f)	Hypertensives	56-73	Dark chocolate	Flavonoids	6.3 g (30 mg)	18 weeks	SBP DBP	↓2.9 mmHg ↓1.9 mmHg

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Table I. (Continued). Effects of food polyphenols on blood pressure in human intervention studies

References	*Type of study*	*No. Ind. †*	*Subjects' characteristics*	*Age range years*	*Substance given*	*Main polyphenols ‡*	*Dose/d (amount of polyphenols)*	*Duration*	*Biomarkers*	*Main changes on BP¥*
Fukino et al. (2008) (*27*)	Chronic, single arm, no control	60 (49m,11f)	Healthy	32-73	Green tea-extract powder	Catechins	1 packet (544 mg)	2 months	DBP	↓4 mmHg
Wilson et al.(2008) (*28*)	Acute, controlled parallel	187 (38m,149f)	Healthy	19-20	Cranberry extract or other beverage	Anthocyanins, phenolic acids	480 mL	Postprandial	BP Heart rate (from 0 to 180min)	↔ ↔
Borochov-Neori et al.(2008) (*29*)	Chronic, single arm, no control	10	Healthy	All ages (adults)	Marula juice	Hydrolysable tannins, catechins	200 mL (56 mg/dL)	21 days	BP	↔
Erlund et al.(2008) (*30*)	Chronic, controlled crossover	71 (25m,46f)	Subjects with CVD risk factors	51-64	Bilberries, lingonberries, blackcurrant, strawberry puree and raspberry juice	Anthocyanins	150 g (837 mg)	56 days	SBP	↓ by 7·3mmHg in subjects with high baseline BP in the treatment group

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References	*Type of study*	*No. Ind. †*	*Subjects' characteristics*	*Age range years*	*Substance given*	*Main polyphenols ‡*	*Dose/d (amount of polyphenols)*	*Duration*	*Biomarkers*	*Main changes on BP¥*
Monagas et al. (2009) (*31*)	Chronic, controlled crossover	42 (19m,23f)	Healthy	58-81	Cocoa powder (with skim milk)	Flavonoids	40 g (495.2 mg)	28 days	SBP DBP Heart rate	↔ ↔ ↔
Morand et al. (2010) (*32*)	Chronic, controlled crossover	44 (all men)	Healthy overweight	50-65	Orange juice or hesperidin enriched drink.	Flavonoids (Hesperidin)	500 mL (342 mg)	28 days	SBP DBP Pulse pressure	↔ ↓3.2-5.5 mmHg ↔
van Mierlo et al. (2010) (*33*)	Chronic, controlled crossover	35 (all men)	Healthy	18-45	Wine grape or grapes seed extracts (capsules)	Anthocyanins, phenolic acids	6 capsules (800 mg)	14 days	SBP DBP Heart rate	↔ ↔ ↔

SBP, systolic blood pressure; serum ACE, plasma angiotensin 1-converting enzyme; DBP, diastolic blood pressure; BP, blood pressure.* Table is arranged by year in ascending order. † m: male, f: female ‡ Only the top two polyphenols with the highest concentrations are listed. ¥ ↑refers to increase; ↓ refers to decrease; ↔ refers to no change; unless otherwise stated, (%) refers to changes from baseline when test substance given.

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Table II. Possible interferences in urine by Folin-Ciocalteu Assay

Reductant compounds (maximum levels in urine)	*F-C Assay*	*F-C post SPE*
Sugars: Glucose (2 mg/L) and Fructose (1 mg/L)	-	-
Fe (II) (1 mg/L)	+	-
Organic acids: Oxalic, Citric and Tartaric acids (100 mg/L)	-	-
Aminoacids: Phe, Tyr, Glut, Arg (1 mg/L)	Weak	-
Vitamin C (100 mg/L)	+	-
Folic acid (100 mg/L)	-	-
Hippuric acid (10 mg/L)	-	-
Epinephrine or adrenaline (0.02 mg/L)	+	-
Norepinephrine or noradrenaline (0.08 mg/L)	+	-
Dopamine (0.4 mg/L)	+	-
Drugs (use)	*F-C Assay*	*F-C post SPE*
Paracetamol (analgesic, antipyretic)	+	+
AAS (analgesic, antipyretic)	-	-
Celecoxib (analgesic, antidysmenorrhoea, antirheumatic, anti-inflammatory)	-	-
Diclophenaco (NSAIDs)	-	-
Ibuprofen (NSAIDs)	-	-
Digoxine (antiarrhytmic, cardiotonic)	-	-
Manidipine (antihypertensive)	-	-
Hidrochlorotiazine (antihypertensive)	-	-
Enalapril Maleate (antihypertensive)	-	-
Losartan (antihypertensive)	-	-
Amlodipino (antihypertensive)	-	-
Atenolol (antihypertensive)	-	-
Doxazocina (antihypertensive)	-	-
Rupatadine (antihistaminic)	-	-
Simvastatine (hypolipidemic)	-	-
Bezafibrate (hypolipidemic)	-	-
Omeprazol (inhibits gastric acidity)	+	-
Alopurinol (reduces uric acid production)	-	-
Glimepiride (oral antidiabetic)	-	-

Continued on next page.

Table II. (Continued). Possible interferences in urine by Folin-Ciocalteu Assay

Drugs (use)	*F-C Assay*	*F-C post SPE*
Metformina (oral antidiabetic)	-	-
Gliclazida (oral antidiabetic)	+	-
Insulina (injectable antidiabetic)	+	+
Paroxetina (anxiolytic)	-	-
Aprazolam (anxiolytic)	-	-
Melatonina (anti jet-lag)	+	-

(+) Substances that react with the F-C. (-) There is no reaction, so this substance does not interfere.

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The aim of the current study was to evaluate the usefulness of a new biomarker (TPE) and to correlate it with BP and the prevalence of hypertension (*17*).

Subjects and Design

The PREDIMED (*PREvención con DIeta MEDiterránea*) study is a large, parallel-group, multicenter, randomized, controlled 5-year clinical trial aimed to assess the effects of the Med-Diet on the primary prevention of cardiovascular disease (www.predimed.org; ISRCTN35739639). The detailed recruitment method and study protocol have been described previously (*5*). From October-2003 to July-2004, we selected 612 potential participants in primary health centers. Eligible participants were community-dwelling men aged 55 to 80 years and women aged 60 to 80 years, who were free of cardiovascular disease at baseline and fulfilled at least one of the following two criteria: (1) type-2 diabetes mellitus and/or (2) three or more coronary heart disease (CHD) risk factors (*39*). The participants provided written informed consent and the study protocol was approved by the Institutional Review Boards of the two participating centres.

Measurements

At baseline, all participants completed a validated semiquantitative FFQ with 136-items (*40*), the validated Spanish version (*41*) of the Minnesota Leisure Time Physical Activity Questionnaire, and a 47-item questionnaire about education, lifestyle, history of illnesses and medication used. Trained nurses measured BP thrice with a validated semi-automatic oscillometer (Omron HEM-705CP (*42*); Hoofddorp, The Netherlands). Energy and nutrient intake was derived from Spanish food composition tables (*43*). TP consumption from plant food and beverages (mg/g fresh matter) was quantified according to Saura-Calixto F *et al.* (*44*) and Brat P *et al.* (*45*), from the data of the FFQ. For TPE determination urine samples were thawed on a ice bed for 3 h; they were centrifuged for 10 min at 4

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ºC and 1 mL of supernatants, catechin and gallic acid standards for calibrated line (1, 2, 4, 6, and 8 mg L^{-1}) were diluted with 1 mL of water Milli-Q and acidified with 34 µL of hydrochloric acid at 35%; they were used to load the Oasis® MAX 96-well plate SPE cartridges separately. The extraction procedure described above for Oasis®MAX cartridges was applied and 15 µL of the eluted fractions were mixed with 170 µL of Milli-Q water in the thermo microtiter 96-well plate (nunc™, Roskilde, Denmark), adding 12 µL of F-C reagent and 30 µL of sodium carbonate (200 g/L). The multichannel pipette minimized differences in the times (3 s) of the F-C reaction between the eight lines of the 96-well plate, ensuring a similar reaction time for all samples analyzed on the same plate. The 96-well plate permitted fewer reagents to be used in a more environmentally friendly test. The mixtures were incubated for 1 h at room temperature in the dark. After the reaction period, 73 µL of Milli-Q water were added with the multichannel pipette. Absorbance was measured at 765 nm in UV/VIS Thermo Multiskan Spectrum spectrophotometers (Vantaa, Finland). This spectrophotometer allowed the absorbance of a 96-well plate to be read in only 10 s.

For creatinine in urine samples, 3 µL of urine were mixed with 60 µL of aqueous picric acid solution (1%) and 5 µL of sodium hydroxide (10%). After shaking, the mixture was left 15 min in the dark at room temperature; 232 µL of Milli-Q water was added and the absorbance was measured at 500 nm in the UV/VIS spectrophotometers. Total polyphenols were expressed as mg gallic acid equivalent (GAE)/ g creatinine and mg catechin/ g creatinine.

Results and Discussion

Table III shows the average food consumption of study participants divided according to quartiles of urinary TPE expressed as mg GAE/g creatinine. Significant increasing trends across quartiles of TPE were observed for the intake of fruits, vegetables, total F&V, dairy products, fish and TP intake; whereas decreasing trends across TPE quartiles were observed for total alcohol intake, cereals, common olive oil, pastries, cakes or sweets, as well as total energy intake.

The linear regression analyses of TPE in spot urine samples and TP intake (100 mg) and total F&V intake (100g) are presented in Table IV with various models after adjusted for potential confounding factors. We observed a significant positive association between urine TPE and daily intake of F&V in the unadjusted model (β=0.131; P<0.001). After adjusting for potential confounding factors the association remained statistically significant in the multivariate regression analysis. The standardized coefficients (Beta) are the regression coefficients obtained with the regression model using the standardized values (measured in standard deviation units), and are therefore independent of measurement units. The standardized coefficients from this model showed that total phenol intake (Beta=0.283) contributed more to urinary TPE than F&V intake (Beta=0.150)

On multivariate linear regression analyses, systolic and diastolic BP exhibited a monotonic inverse association with TPE in spot urine samples (quartiles) after adjustment for potential confounders (Table V). The non-standardized coefficients, β=-1.731, P=0.024 and β=-1.264, P=0.003 represent the expected

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change of systolic and diastolic BP, respectively, corresponding to an increase from a TPE to the upper quartile.

Logistic regression analysis showed an inverse association between urinary TPE (by quartile) and the prevalence of hypertension (Table VI). Compared to the participants in the lowest quartile of TPE (<88.99 mg GAE/g creatinine), participants in the highest quartile (>160.23 mg GAE/ g creatinine) had a significantly reduced prevalence of hypertension (OR=0.71, CI 0.53 to 0.95; *P*=0.022) in the unadjusted model. In all three models, a significant difference was observed between the top and the bottom quartiles for the prevalence of hypertension, after adjustment for potential confounders. When the analysis was adjusted for all possible confounding factors (model 4), participants in the highest quartile had a 36% reduced odds of hypertension (OR=0.64, CI 0.45 to 0.92; *P*=0.015), compared to those in the lowest quartile.

Finally, BP correlated better with urinary TPE than with TP intake assessed by FFQ. In fact, a highly significant association between polyphenol intake assessed via TPE in urine and systolic (*P* for trend = 0.024) and diastolic BP (*P* for trend = 0.003) was observed, whereas polyphenol intake assessed via FFQ tended to be associated with BP values, but here the association did not reach the statistical significance.

The results from the present study provide evidences that total phenol and F&V intake in the Mediterranean diet are positively correlated with the excretion of TPs in spot urine samples. Thus, F&V consumption is the main contributor to urinary TPE.

Polyphenols are the main dietary represent a wide variety of structures from different subclasses. The biological effects of these compounds depend on their bioavailability, their kinetics and exposure time (*46*). The main sources of these compounds are fruit, vegetables and beverages such as wine, coffee and tea which contain complex mixtures of often poorly characterized polyphenols (*47*), possibly explaining the difficulties in determination of total phenol content in foods (*45*).

The most common polyphenols in the human diet are not necessarily the most active *in vivo*, either because they have a lower intrinsic activity than others or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated (*47*).

The presence of multiple antioxidants in fruit- and vegetable-rich diets may explain the lowering effect on BP observed in hypertensive patients. A protect effect of F&V against cardiovascular disease (CVD) has been observed in numerous epidemiological studies (*7*, *8*). Thus, high intake of F&V correlated with a reduced risk of CVD in a study on 2682 men in Finland (*48*). Radhika G. *et al.* (*49*) also examined the relationship between F&V intake and CVD risk factors in urban south Indians. The study population was comprised of 983 individuals aged 20 years or more, selected from the Chennai Urban Rural Epidemiological Study (CURES). Linear regression analysis revealed that after adjusting for potential confounder factors, the highest quartile of F&V intake showed a significant inverse association with systolic BP ($\beta = -2.6$ mmHg; $P = 0.027$), when compared with the lowest quartile. A high intake of F&V explained 48% of the protective effect against CVD risk factors.

Table III. Daily intake of selected foods according to quartiles of excreted total urinary polyphenols, expressed as mg GAE /g creatinine. Reprinted from: Medina-Remón A, *et al.* (*17*). Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, Nutr Metab Cardiovasc Dis (2009), doi:10.1016/j.numecd.2009.10.019 Copyright 2009, with permission from Elsevier

	Urine mg GAE/ g creatinine concentration quartile				
	Q1 (<89.0)	*Q2 (89.1-119.5)*	*Q3 (119.6-160.2)*	*Q4 (>160.3)*	*P for trend*[1]
Urine total polyphenol (mg GAE/ g creatinine)[2]	72.8 (11.6)	103.1 (8.2)	138.2 (11.1)	226.1 (69.8)	< 0.001
No. of subjects	147	147	147	147	0.742
Olive oil (g)	48.7 (14.3)	47.3 (13.7)	45.1 (14.6)	45.0 (15.7)	0.014
Total nuts (g)	8.6 (10.7)	9.2 (11.3)	9.6 (12.2)	9.1 (10.9)	0.680
Vegetables (g)	253.1 (80.2)	261.1 (81.1)	262.6 (84.0)	272.7 (92.1)	0.053
Legumes (g)	17.9 (7.0)	17.9 (7.5)	17.7 (7.0)	16.5 (7.6)	0.107
Fruits (g)	304.6 (143.6)	319.3 (141.3)	315.1 (132.9)	360.8 (151.2)	0.002
Total fruits and vegetables (g)	557.7 (176.8)	580.4 (173.3)	577.7 (162.1)	633.5 (190.1)	0.001
Fish or seafood (g)	86.5 (34.0)	82.2 (35.8)	88.7 (36.9)	94.1 (39.0)	0.030
Meat or meat products (g)	132.0 (49.6)	129.7 (43.7)	134.3 (48.5)	134.2 (47.5)	0.531
Pastries, cakes or sweets (g)	34.8 (31.6)	27.9 (24.8)	25.3 (25.9)	23.7 (28.7)	< 0.001
Cereals (g)	243.7 (106.7)	235.8 (91.6)	228.7 (98.3)	211.3 (83.3)	0.003
Milk and dairy products (mL)	352.1 (214.8)	378.8 (216.5)	396.5 (222.1)	403.1 (215.1)	0.033
Wine (mL)	121.1 (147.0)	81.5 (137.9)	101.1 (161.5)	81.3 (149.3)	0.071
Coffee (mL)	63.3 (47.7)	69.6 (52.9)	67.5 (48.3)	74.5 (56.0)	0.097
Tea (mL)	3.92 (16.4)	3.25 (11.4)	4.16(12.6)	5.47 (20.4)	0.332
Chocolate (g)	3.1 (6.2)	2.4 (5.2)	2.3 (5.5)	1.9 (4.8)	0.063
Natural orange juice (mL)	23.3 (54.3)	25.19 (57.8)	13.4 (41.2)	19.1 (50.8)	0.199

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Table III. (Continued). Daily intake of selected foods according to quartiles of excreted total urinary polyphenols, expressed as mg GAE /g creatinine.

	Urine mg GAE/ g creatinine concentration quartile				
	Q1 (<89.0)	*Q2 (89.1-119.5)*	*Q3 (119.6-160.2)*	*Q4 (>160.3)*	*P for trend*[1]
Total polyphenol intake (mg GAE)	1075.6 (354.9)	1057.5 (320.2)	1086.2 (322.3)	1222.5 (439.8)	0.001
Alcohol (g)	15.7 (17.9)	10.2 (16.0)	12.2 (18.5)	9.9 (17.8)	0.018
Fibre (g)	22.1 (6.2)	22.0 (5.5)	21.9 (5.2)	22.5 (6.2)	0.606
Cholesterol (g)	353.6 (119.7)	328.6 (93.7)	340.5 (113.4)	342.1 (89.4)	0.561
Sodium (mg/d)	3347.7 (959.2)	3088.0 (905.4)	3123.2 (1006.5)	3145.4 (877.2)	0.100
Potassium (mg/d)	3926.7 (722.3)	3929.4 (700.9)	3994.8 (805.5)	4029.7 (659.7)	0.161
Total energy, Kcal/d	2380.1 (586.8)	2238.0 (472.0)	2205.1 (547.4)	2138.5 (476.8)	< 0.001

[1] One-factor ANOVA was used for continuous variables and χ^2-test for categorical variables; [2] Mean (standard deviation). GAE: gallic acid equivalent.

In the Nurses' Health Study, intake of F&V was also inversely associated with systolic and diastolic BP, whereas the intake of cereals and meat was directly associated with systolic BP (*50*). In the Chicago Western Electric Study vegetable protein, beta-carotene, and an antioxidant vitamin score based on vitamin C and beta-carotene were inversely and significantly related to an average annual change in BP, after the 8-year follow-up in 1714 employed middle-aged men (*51*). On the other hand, Hung HC *et al.* (*52*) evaluated the association of F&V consumption with peripheral arterial disease in a cohort of 44,059 men initially free of cardiovascular disease and diabetes, reporting no evidence that F&V consumption protects against peripheral arterial disease. In the age-adjusted model, men in the highest quintile of F&V had a relative risk of 0.55 (95% CI = 0.38-0.80) for peripheral arterial disease, compared with those in the lowest quintile. However, the associations were greatly weakened after adjustment for smoking and other traditional cardiovascular risk factors. .

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Table IV. Multivariate linear regression analysis with total polyphenol excreted normalized, in spot urine samples (mg GAE/g creatinine) as the dependent variable, and total polyphenol (100mg/d) and total fruit and vegetable (100g/d) as exposure variable, adjusted for potential confounders. Reprinted from: Medina-Remón A, *et al.* (*17*). Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, Nutr Metab Cardiovasc Dis (2009), doi:10.1016/j.numecd.2009.10.019 Copyright 2009, with permission from Elsevier

	Model	*β*	*SE*	*Beta*	*P*	*95% CI*
Total polyphenol intake (100mg/d)	Model 1	0.073	0.017	0.179	<0.001	0.041 to 0.106
	Model 2	0.110	0.016	0.268	<0.001	0.078 to 0.141
	Model 3	0.117	0.017	0.286	<0.001	0.085 to 0.150
	Model 4	0.116	0.020	0.283	<0.001	0.077 to 0.154
Fruit and Vegetables (100g)	Model 1	0.131	0.035	0.155	<0.001	0.064 to 0.199
	Model 2	0.112	0.033	0.132	0.001	0.047 to 0.177
	Model 3	0.107	0.034	0.126	0.002	0.040 to 0.174
	Model 4	0.127	0.036	0.150	<0.001	0.056 to 0.198

β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; *P*: two-sided test of significance; Model 1: unadjusted; Model 2 was adjusted by sex, age and weight; Model 3 as in Model 2 plus smoking status, physical activity, educational level and energy expenditure in physical activity; Model 4 was adjusted as in Model 3 plus total fish/seafood, olive oil, beer, chocolate, natural orange juice, tea, cereals, milk and dairy products, meat or meat products, nuts, pastries/cakes/sweets, fruits and vegetables, wine and coffee intake; GAE: Gallic acid equivalent.

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Table V. Multivariate linear regression analyses with systolic blood pressure and diastolic blood pressure as the dependent variables, and quartile of total polyphenol excreted in spot urine samples (mg GAE/g creatinine) as exposure variable, adjusted for potential confounders. Reprinted from: Medina-Remón A, *et al.* (*17*). Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, Nutr Metab Cardiovasc Dis (2009), doi:10.1016/j.numecd.2009.10.019 Copyright 2009, with permission from Elsevier

	Model	*β*	*SE*	*Beta*	*P*	*95% CI*
Systolic blood pressure	Model 1	-1.743	0.712	-0.104	0.015	-3.141 to -0.345
	Model 2	-1.895	0.741	-0.113	0.011	-3.350 to -0.440
	Model 3	-1.895	0.743	-0.113	0.011	-3.354 to -0.436
	Model 4	-1.731	0.765	-0.103	0.024	-3.233 to -0.228
Diastolic blood pressure	Model 1	-1.705	0.397	-0.180	<0.001	-2.485 to -0.925
	Model 2	-1.438	0.408	-0.152	<0.001	-2.238 to -0.637
	Model 3	-1.405	0.409	-0.148	0.001	-2.208 to -0.602
	Model 4	-1.264	0.422	-0.133	0.003	-2.092 to -0.435

β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; *P*: two-sided test of significance; Model 1: unadjusted; Model 2: adjusted by sex, age and weight; Model 3 adjusted as in Model 2 plus smoking status, physical activity, and educational level; Model 4 adjusted as in Model 3 plus drug consumed in the last month, sodium and potassium intake and glomerular filtration rate (GFR); GAE: Gallic acid equivalent.

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Table VI. Multivariate adjusted odds ratios (95% confidence intervals) for cardiovascular risk factors (hypertension) according to quartiles of total polyphenol excretion expressed as mg GAE/ g creatinine using the lowest quartile group as reference category. Reprinted from: Medina-Remón A, *et al.* (*17*). Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, Nutr Metab Cardiovasc Dis (2009), doi:10.1016/j.numecd.2009.10.019 Copyright 2009, with permission from Elsevier

	Urine mg GAE/ g creatinine concentration quartile				
	Q1 (<88.99)	*Q2 (89-119.46)*	*Q3 (119.47-160.22)*	*Q4 (>160.23)*	*P for trend*
Hypertension, n (%)	123 (83.7)	126 (85.1)	113 (76.9)	114 (77.6)	0.067
Model 1	1.00	1.82 (0.33-10.13)	0.67 (0.29-1.55)	0.71 (0.53-0.95)	0.021
Model 2	1.00	1.40 (0.21-9.24)	0.61 (0.25-1.47)	0.64 (0.46-0.89)	0.006
Model 3	1.00	1.29 (0.19-8.49)	0.55 (0.22-1.37)	0.65 (0.47-0.91)	0.007
Model 4	1.00	1.39 (0.19-10.34)	0.55 (0.20-1.48)	0.64 (0.45-0.92)	0.047

Model 1, unadjusted; Model 2 was adjusted for sex, age and weight; Model 3 adjusted as in Model 2 plus smoking status, physical activity, educational level and energy expenditure in physical activity; Model 4 was adjusted as in Model 3 plus medication intake: ACE inhibitor, diuretics, statins (hypolipidemic drugs), insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month, sodium and potassium intake and glomerular filtration rate (GFR); GAE: Gallic acid equivalent.

All these epidemiological evidence support the hypothesis that a diet rich in F&V may prevent BP from increasing and may help to decrease elevated BP levels. However, only one study (*17*) has attempted to correlate the biomarker of TP intake, determined in spot urine samples, with BP measurements or with the prevalence of hypertension. Taking into account that greater excretion of polyphenols in urine is determined by high TP consumption, we suggested that the inverse association observed between the objectively measured TPE in urine samples with BP may be related to a favorable effect of TP intake on preventing raised BP levels.

The BP lowering effects of fruit- and vegetable-rich diets in hypertensive patients have mainly been attributed to the presence of multiple polyphenols in these foods. In respect to the last point, some studies have suggested that polyphenols contained in foods may exert antihypertensive effects and contribute to the prevention of hypertension, due to its vasodilatation properties related to the increasing release of endothelial-derived nitric oxide (*53*, *54*).

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Conclusions

Total polyphenol consumption (TPE) can be used as a polyphenol intake biomarker. It has been found a negatively association between high TPE in urine samples and BP levels in an elderly Mediterranean population (*17*). Thus, in order to lower cardiovascular risk, which is associated to high blood pressure, a high intake of polyphenol-rich diet mainly from fruits and vegetables is recommended. On comparing the participants in the highest with those in the lowest quartile of TPE a 36% reduction was observed in the odds ratio of hypertension. However, the observation that systolic and diastolic BP decreases when TPE increases should be confirmed in further intervention studies.

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Chapter 27

The Pig Cecum Model – A Powerful Tool for Studying the Intestinal Metabolism of Flavonoids

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Experimental studies using animals or cultured human cell lines support a role of polyphenols in the prevention of cardiovascular and neurodegenerative diseases, cancer, diabetes and osteoporosis. Basic principle is supposed to be their antioxidative potential (*1–3*). However, the data on the bioavailability of polyphenols are very limited and mainly based on the analysis of blood, urine or fecal samples. There is only little information available regarding the intestinal absorption and metabolism by the gut microbiota (*4–6*). For this reason different *in vitro*-systems have been developed studying the metabolism by the intestinal bacteria. However most *in vitro*-experiments are not comparable with *in vivo*-conditions. Incubation with single bacterial species represents only a small section of the complex gut microbiota (*7*, *8*). The use of fecal samples is critical due to air contact while excretion (*9*). For the pig cecum model, the microbiota is isolated from the cecum of freshly slaughtered pigs under strict anaerobic conditions. The comparability between humans and pigs is shown by the fluorescence *in situ* (FISH)-technique with 16S rRNA based oligonucleotide probes (*10*).

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Introduction

Flavonoids belong to the class of polyphenols. This group of food constituents is highly observed by consumers due to attributed health effects. Moderate wine consumption is correlated with a prevention of coronary heart diseases (*11*). Several forms of cancer occur less often in cohorts drinking tea and an overall high intake of fruits and vegetables may reduce the risk of getting a stroke (*12*, *13*). In the 1990's these information led to the very popular "Mediterranean diet", now extended by some Asian influences from soy and green tea.

Flavonoids are secondary plant metabolites and mainly found in higher plants, e.g. fruits, vegetables or tea. Data about the daily intake of flavonoids vary from 20 mg/day to 1 g/day (*14–16*) depending on individual dietary habits. Most flavonoids occur in plants as *O*-glycosides. The linkage of different sugar moieties stabilizes the aglycones and increases their water solubility (*17*, *18*). More seldom are the *C*-glycosides with high amounts in rooibos tea (*19*, *20*), but also being generated by food processing (*21*, *22*).

Several studies have shown flavonoids to be potent antioxidants and radical scavengers in different model systems (*3*, *23*). These results led to an assumption of protective effects by flavonoids. Requirement for these physiological effects, except from some local ones, would be the absorption into the bloodstream. Polyphenols resist the acidic conditions in the stomach, but little hydrolysis may occur in the small intestine (*24*, *25*). The stability of procyanidins during gastrointestinal passage is high; there is no degradation in the stomach (*25*). In the small intestine only slight degradation seems to take place (*26*). To overcome the intestinal barrier the necessity of previous cleavage of the glycosides (*27*, *28*) is discussed as vividly as the possibility of an active transport of intact glycosides by the sodium dependent glucose transporter 1 (SGLT1) (*29–31*). The resorption of flavonoid *C*-glycosides is characterized even worse, but most of the compounds should reach the proximal colon (*30*, *32*). The absorption of procyanidins is mechanistically unclear and quantitatively even lower than the absorption of flavonoid monomers (*33*, *34*). Food constituents which are not degraded by enzymes and/or absorbed are summed up as roughage and can intensively be metabolized by intestinal microorganisms.

The Pig Cecum Model

Pigs are well accepted and used animals for modeling and studying human metabolism and digestive physiology (*35*, *36*). Regarding size, type of diet as well as amount and rate of ingestion they are of all animals most similar to humans. For example pigs are the only usual laboratory animals having a haustrated colon (*37*, *38*).

But there are also some differences in anatomy. Humans have nearly the same pH value at every part of the stomach, while pigs have a combined stomach. This may yield in higher amounts of *lactobacilli* in the ileum (*36*, *39*). The ratio between the overall size and the length of the intestine is not equal as pigs are bred herbivorous (*39*). Anyway, as the major ingredients of the feed are metabolized equally Donaldson *et al.* assumed in the late 1960's, that the microbiota should be comparable (*40*).

For the pig cecum model, the pigs (German Landrace or Angler Sattel x Pietrain) from which the ceca were obtained were raised by biodynamic farming. They were 10-12 months old and weighted 120-150 Kg. They were fed with a basal diet composed of rye, spelt, linseed, lentil, corn, millet, and rice enhanced with clover silage.

The ceca were ligated and culled during slaughtering and then handled under anaerobic conditions using an anaerobic jar (Merck, Darmstadt, Germany). Furthermore, all preparations and experiments with the inoculum were performed in an anaerobic chamber flushed with carbon dioxide to retain an anaerobic atmosphere. The inocula of each cecum were isolated and suspended in the same volume of phosphate buffered saline (pH 6.4) containing a trace element solution. All solutions, buffers, and vessels were flushed with a mixture of nitrogen and carbon dioxide before use. Removal of large particles from the inoculum suspension was accomplished by filtration through a coarsely meshed net. These filtrates were used for the incubation experiments. Sterilized control samples and blank samples were used as control for chemical degradation and matrix effects (*41–43*).

The suitability of the pig cecum model was shown by the comparison of porcine with human microbiota using fluorescence *in situ* hybridization (FISH)-technique and counting under an epifluorescence microscope. Therefore, distinct parts of the 16S ribosomal RNA are used as linkage points for probes. Five oligonucleotide probes were applied for the detection of bacterial 16S ribosomal RNA of the genus *Bifidobacterium* (Bif164 (*44*)) and of different phylogenetic cluster of *Enterococcus* (Lab158 (*45*)) *Bacteroides* (Bac303 (*46*)), *Eubacterium*, and *Clostridium* species (Lowgc2P, Erec482 (*47*)). Total bacteria counts in the cecal samples were enumerated by a *Bacteria* domain-specific probe (Eub338, (*10*)). Probe Eub338 was labeled at its 5' end with fluorescein, all the other probes were coupled with carbocyanin (*48*). The results show large interindividual differences between the donors, which are at least as huge as the interspecial differences between humans and pigs (Figure 1; 2).

The total numbers of colony forming units for each group of microorganism are comparable in pig and men (*48*), what is supported by findings in literature according to both species (*49*, *50*).

In general interindividual differences in microbiota composition are caused by nutrition, host-microorganism cross talk and interaction between different microorganisms (*51–53*). For this reason a single incubation experiment with later combination of the data has been preferred in contrast to a pooling of samples, because this approach reflects the *in vivo* situation much better.

Degradation of Anthocyanins

Anthocyanins are a group of polyphenols with about 580 single structures based on 32 naturally occurring anthocyanidins (*54*). In the plants anthocyanins act as attractors, natural sunscreen and antioxidants or radical scavengers. Red cabbage, various berries and wine are rich in anthocyanins and contain up to 600 mg/100 g (*55*, *56*). North-Americans consume ca. 12.5 mg anthocyanins with their daily diet (*57*), while other authors assume up to the 20-fold amount (*16*, *58*).

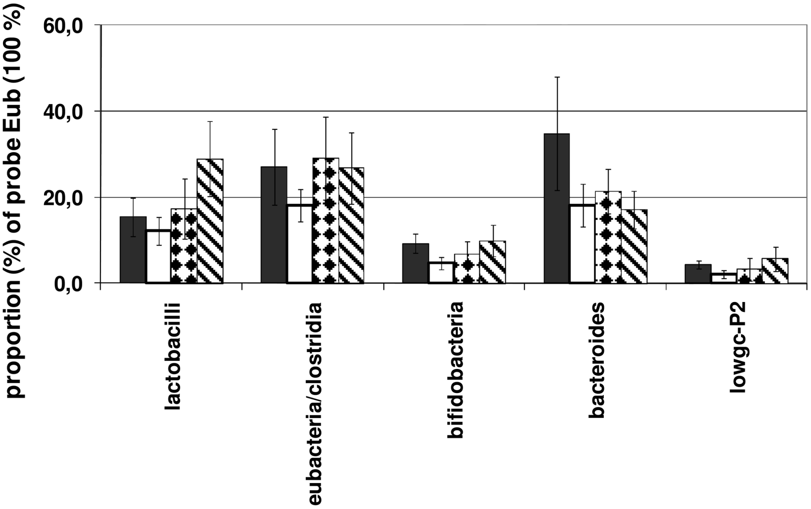


Figure 1. Results of the enumeration of microorganisms of different pig ceca by FISH-technique (n = 4) (48).

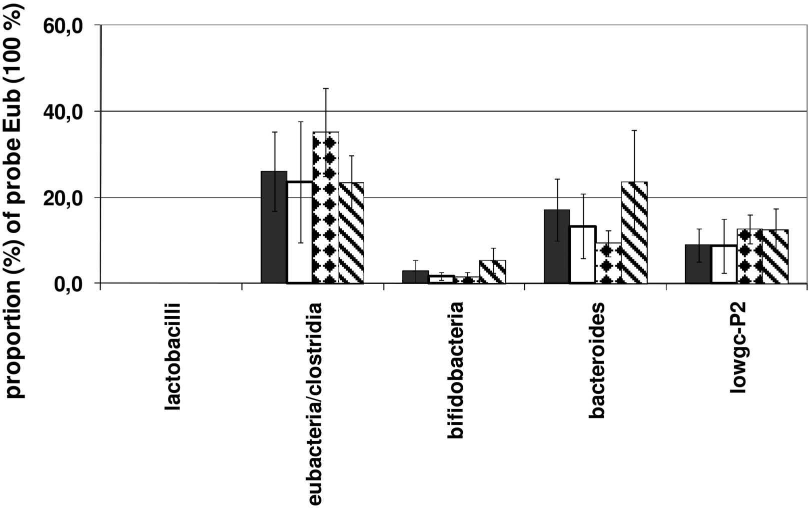


Figure 2. Results of the enumeration of microorganisms of human volunteers by FISH-technique (n = 4) (47).

The six anthocyanins cyanidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside, malvidin-3-rutinoside, malvidin-3,5-diglucoside and cyanidin-3,5-diglucoside have been studied in the pig cecum model (*42*). All glycosides are cleaved into phloroglucinolaldehyde and a hydroxylated phenylcarboxylic acid between 20 min and 2 h as can be exemplarily seen for malvidin-3-glucoside in Figure 3.

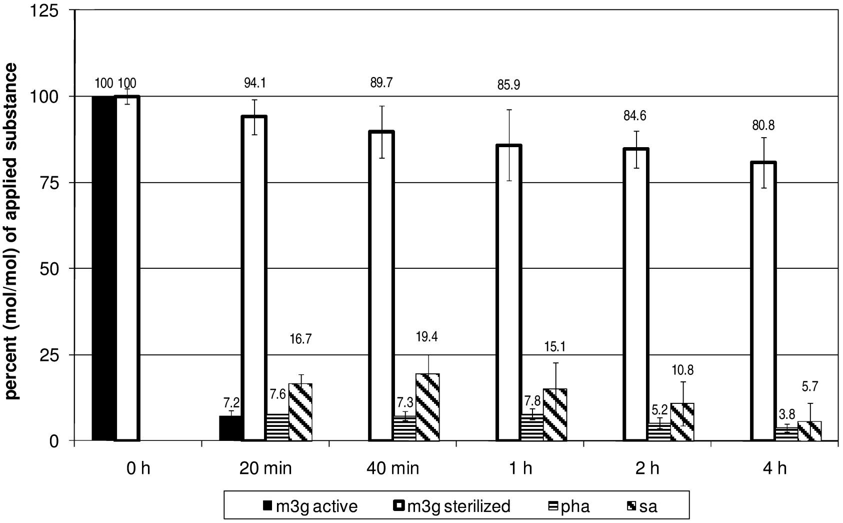


Figure 3. Relative proportions (% (mol/mol)) of malvidin-3-glucoside (m3g) and its degradation products phloroglucinolaldehyde (pha) and syringic acid (sa) in active cecal suspension in reference to that in sterilized inoculum (n = 6, mean ± SD; measured by HPLC-UVD and GC-MS). Data are normalized for a recovery of 100% (mol/mol) at 0 h. (42)

The liberated aglycones have been detectable in amounts below 10 µM in the samples. Obviously the secondary degradation of the aglycones is faster than microbial cleavage of the *O*-glycosidic bonds. Further experiments with free anthocyanidins showed the same fast degradation comparable to liberated anthocyanidins in this experiment. Results from the sterilized control samples show the stability of the glycosides under physiological conditions without viable microbiota (Figure 3, m3g sterilized) in opposition to an incubation of the free aglycones.

According to the substitution pattern different degradation products are formed. Due to the cleavage in the C-ring of the anthocyanidins phloroglucinolaldehyde (pha) is the product out of the former A-ring. The former B-ring yields protocatechuic acid if cyanidin is incubated; syringic acid in the case of malvidin and vanillic acid as degradation product of peonidin. This reflects exactly the former functional groups of the intact flavonoid as can be seen from Figure 4. The results are in agreement with literature findings of similar experiments (*59–61*).

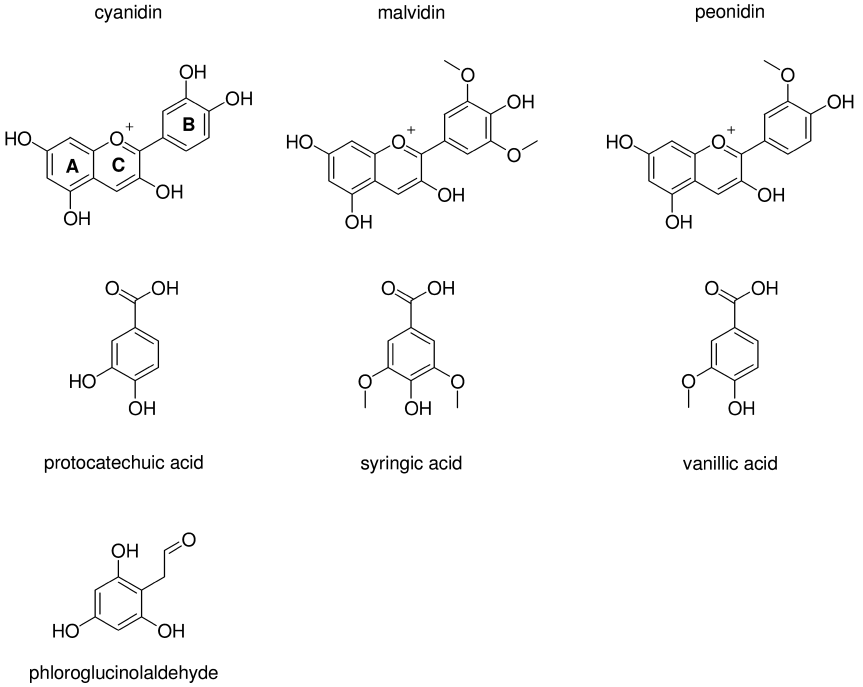


Figure 4. Applied anthocyanidinic structures and degradation products.

The resistance against degradation was cyanidin-3-glucoside = malvidin-3-glucoside > peonidin-3-glucoside > cyanidin-3,5-diglucoside = malvidin-3,5-diglucoside >> cyanidin-3-rutinoside.

The chemical degradation of the anthocyanidins can be explained via a pH-dependent turnover to an α-diketone (*61*, *62*). Interestingly, all found results are not only valid for single incubation experiments, but have been confirmed by experiments by FORESTER and WATERHOUSE, where an anthocyanin mixture in a Cabernet Sauvignon extract was degraded in the same way (*63*). For these experiments they solely used freeze-preserved cecal samples (*64*).

Degradation of Flavonols

The recently released database www.phenol-explorer.eu (*65*, *66*) lists 74 flavonols. Due to a better water solubility and stability they occur as glycosides (*17*, *18*) in nearly every higher plant. Data about the daily intake of flavonoids vary from 20 mg/day to 1 g/day (*15*, *16*, *58*) depending on the different food pattern.

With regard to the experiments with anthocyanins the interest in the influence of the sugar, the aglycones, and its type of bond several flavonolglucosides have been studied in the pig cecum model. Namely these have been quercetin-3-glucoside (q3glu), -galactoside (q3gal) and

–rhamnoside (q3rham) (Figure 5; 6) as well as the triglycosides quercetin- and kaempferol-3-*O*-[α-L-dirhamnopyranosyl-(1→2)-(1→6)-β-D-glucopyranoside (Figure 7; 8). Finally, the degradation of quercetin-3-glucoside has been compared with a feruloylglucoside and vitexin, a *C*-glycoside of the flavone apigenin (Figure 9; 10) (*48*).

The degradation rate mostly depends on the hydroxylation pattern of the sugar. If all hydroxyl groups are in the equatorial position degradation is fastest as can be seen in Figure 6. For example after 20 min of incubation only 2.5 ± 2.2% (mol/mol) of quercetin-3-glucoside are detectable, whereas 36.4 ± 6.9% (mol/mol) and 74.5 ± 7.7% (mol/mol) of quercetin-3-galactoside and -rhamnoside are left, respectively. The galactoside with an axial hydroxyl group on *C*4 and the rhamnoside are degraded more slowly; in the latter case especially due to the methyl group at position 6 (Figure 5) (*48*).

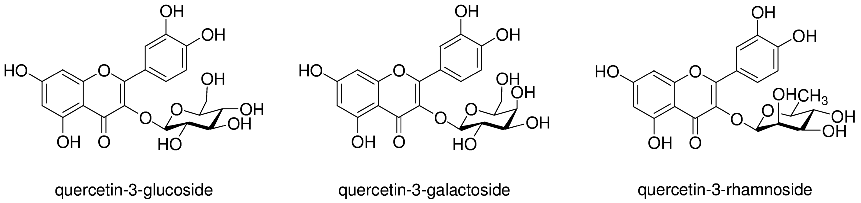


Figure 5. Structures of applied flavonolglycosides.

Degradation of compounds by intestinal microbiota is part of their metabolism and is used for the generation of energy. The microbiota uses the most easy accessible energy source first. This is the reason why the degradation of naturally more widespread occurring flavonoids is faster than that of uncommon ones (*43*). For different sugars this phenomenon was also observed by AURA *et al.* (*67*).

Studying the degradation of two trisaccharides (Figure 7) from red currant (*68*), it becomes clear, that marginal differences in the aglycone structure have less influence on degradation compared to similar differences in the bound sugar moiety.

As can be seen from Figure 8, the microbial degradation of the two flavonoltriglycosides quercetin- and kaempferol-3-*O*-[α-L-dirhamnopyranosyl-(1→2)-(1→6)-β-D-glucopyranoside is in close resemblance. After an incubation time of 1 h, the degradation reached nearly 50% (mol/mol) and within 4 h of incubation both triglycosides were fully hydrolyzed. In accordance to SIMONS *et al.* 2005, the variation of the structure within a subgroup has no influence on the microbial degradation rate, if the 5,7,4’-hydroxylation pattern is still complete (*69*). These results were also confirmed by findings from the comparison of rutinosides of quercetin and cyanidin, which are degraded between similar time points (*48*, *64*).

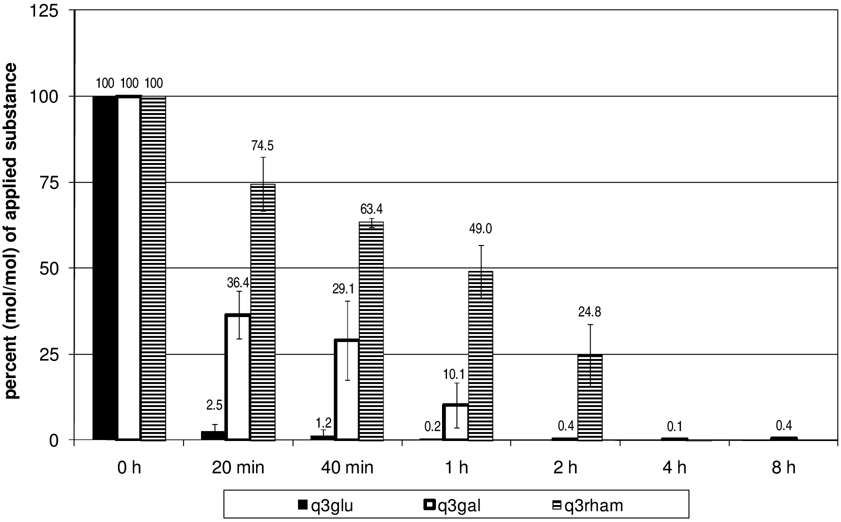
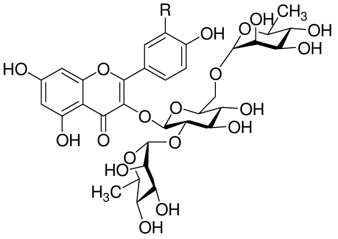


Figure 6. Relative proportions (% (mol/mol)) of quercetin-3-glucoside (q3glu), quercetin-3-galactoside (q3gal) and quercetin-3-rhamnoside (q3rham) in active cecal suspension in reference to that in sterilized inoculum (n=6, mean ± SD; measured by HPLC-UVD). Data are normalized for a recovery of 100% (mol/mol) at 0 h. (48)



R = OH: quercetin-3-O-[alpha-L-dirhamnopyranosyl-(1->2)-(1->6)-beta-D-glucopyranoside
R = H: kaempferol-3-O-[alpha-L-dirhamnopyranosyl-(1->2)-(1->6)-beta-D-glucopyranoside

Figure 7. Structures of applied flavonoltriglycosides.

The degradation of flavonoids depends very much on the bound saccharide. Different glycosides do also show other types of bonds, which again may influence the degradation. The largest group are the *O*-glycosidic bound flavonoids, but there are *C*-glycosides described as well (*19*, *20*). Within the polyphenolic group of the hydroxylated phenylcarboxylic acids ester bonds can also be found. This leads to the question how these compounds are degraded by the intestinal microbiota. Therefore quercetin-3-glucoside, apigenin-8-*C*-glucoside (vitexin), and a feruloylglucoside (Figure 9) have been incubated in the pig cecum model.

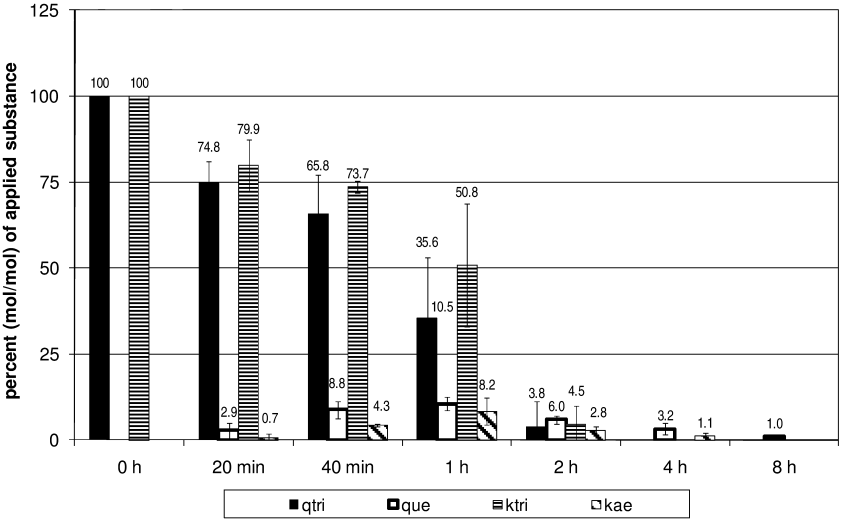


Figure 8. Relative proportions (% (mol/mol)) of quercetin-3-O-[α-L-dirhamnopyranosyl-(1→2)-(1→6)-β-D-glucopyranoside (qtri), kaempferol-3-O-[α-L-dirhamnopyranosyl-(1→2)-(1→6)-β-D-glucopyranoside (ktri) in active cecal suspension with their hydrolyzation products quercetin (que) and kaempferol (kae) (n=6, mean ± SD; measured by HPLC-UVD). Data are normalized for a recovery of 100% (mol/mol) at 0 h. (48)

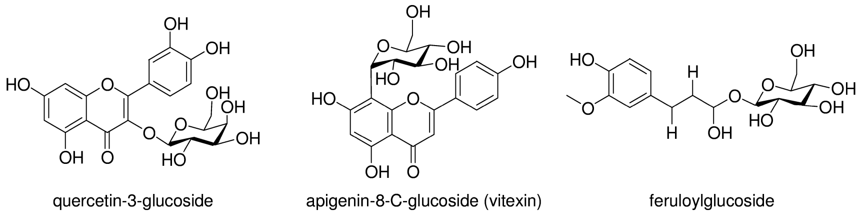


Figure 9. Applied polyphenolic compounds with different bond types.

Varying enzyme activities expressed by the microbiota led to differing degradation times. Quercetin-3-glucoside was completely deglycosylated within 1 h of incubation as described above. The hydrolysis of the ester feruloylglucoside was much faster. Already after 20 min of incubation no intact compound could be detected via HPLC-DAD. The apigenin-8-*C*-glycoside was much more resistant to the degradation caused by the microbiota. After 1 h 84.7 ± 4.2% (mol/mol) were still detectable. Finally it was completely metabolized between 4 and 8 h of incubation time.

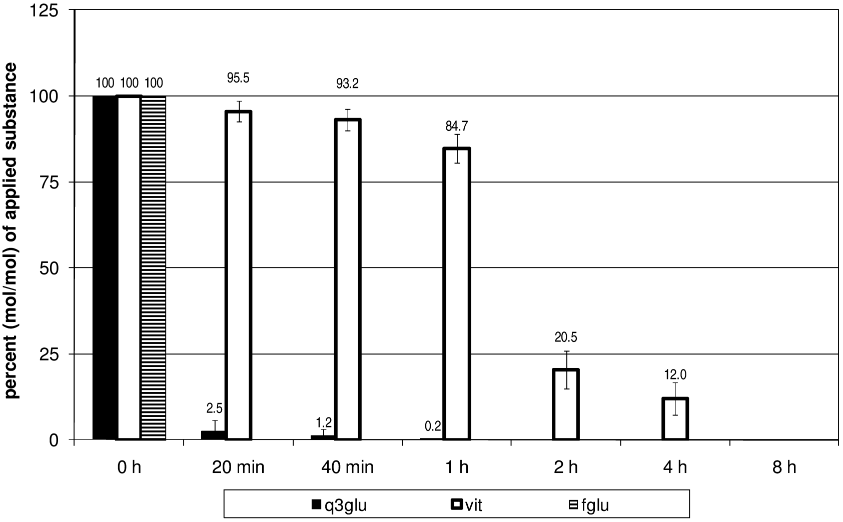


Figure 10. Relative proportions (% (mol/mol)) of quercetin-3-glucoside (q3glu), apigenin-8-C-glucoside (vitexin) (vit) and feruloylglucoside (fglu) in active cecal suspension (n=6, mean ± SD; measured by HPLC-UVD). Data are normalized for a recovery of 100% (mol/mol) at 0 h. (48)

These results indicate a substantial influence of the type of glycosidic bond on the degradation rate of flavonoid glycosides. This finding was additionally confirmed by another incubation experiment with a mixture of following compounds. Four flavonols, namely quercetin-3-rutinoside, -glucoside, -galactoside, and the aglycone quercetin, were mixed with apigenin-8-*C*-glucoside (vitexin) and incubated with 100 µM each. The hydrolysis of the *O*-glycosides was not influenced and therefore comparable to single-incubation experiments. The degradation of applied and liberated quercetin was also in the expected timescale, even if amounts up to 165.0 ± 36.9 µmol/L were detected between 1 and 4 h incubation time (Figure 11).

In contrast to findings of the degradation of apigenin-8-*C*-glucoside (vitexin) was more slowly compared to the single incubation experiment. Whilst in the latter experiment the *C*-glucoside was completely degraded between 4 and 8 h, in mixed incubation only 11.7 ± 2.9% (mol/mol) were still accessible for uptake through the intestinal barrier (*48*). This notice becomes featured by comparison of concentration-time courses for quercetin and apigenin-8-*C*-glucoside (vitexin) at time points of 4 and 8 h. The C-ring cleavage of quercetin takes part faster than hydrolysis of the *C-C* bound sugar moiety.

Degradation of Flavan-3-ols

While most flavonoids are glycosylated, the flavan-3-ols are not, instead galloylation can be found (*18*). Flavan-3-ols do not consist of many basic structures but are the source of many oligo- and polymers. Another remarkable property is the existence of anomeric *C*-atoms at positions 2 and 3, which is shown in Figure 12. These characteristics make flavan-3-ols unique.

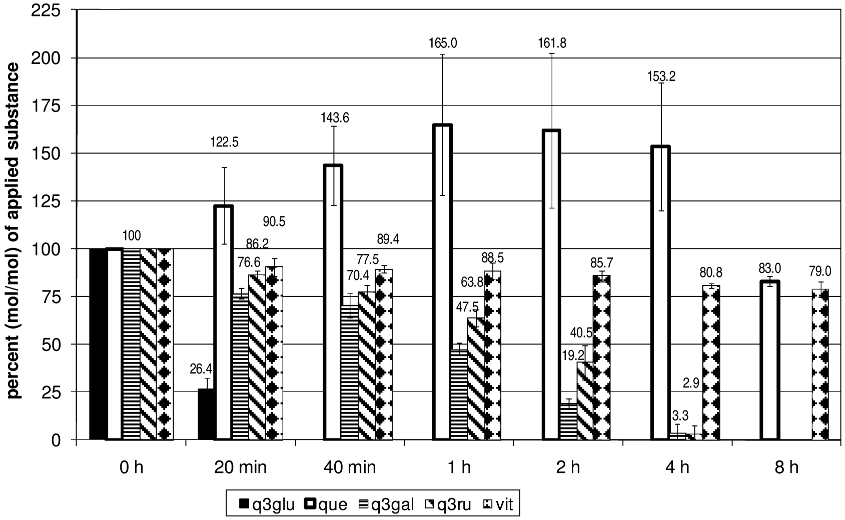
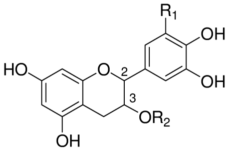


Figure 11. Relative proportions (% (mol/mol)) of quercetin-3-glucooside (q3glu), quercetin-3-galactooside (q3gal), quercetin-3-rutinoside and apigenin-8-C-glucoside (vitexin) (vit) in active cecal suspension with applied and liberated aglycone quercetin (que) (n = 6, mean ± SD; measured by HPLC-UVD). Data are normalized for a recovery of 100% (mol/mol) at 0 h. (48)



R_1	R_2	2	3	substance
H	H	R	S	(+)-catechin
		S	R	(-)-catechin
		S	S	(-)-epicatechin
OH	H	S	R	(-)-gallocatechin
		S	S	(-)-epigallocatechin
H	gallic acid	S	R	(-)-gallocatechin gallate
		S	S	(-)-epigallocatechin gallate

Figure 12. Index of flavan-3-ols.

The flavan-3-ols are favored in foods and beverages like tea, chocolate, or red wine for the oral sensation of astringency (*70*, *71*). But they also got into the focus because of their possible health related effects on antioxidative capacity and radical scavenging activities like other polyphenols (*23*).

(+)-Catechin, one of the most abundant flavan-3-ols, is degraded to similar products as quercetin (*64*). In detail intestinal degradation generated 3,4-dihydroxyphenylpropionic acid (3,4-hppa) as the main intermediary metabolite with 3.6 ± 1.5% (mol/mol) after 20 min and 7.3 ± 2.7% (mol/mol) after 40 min. However 3,4-dihydroxyphenylpropionic acid is further metabolized by the microbiota and is no longer detectable than 1 h. The release of 4-hydroxybenzoic acid (4-hba) was nearly linear to the timepoint of 2 h with 25.4 ± 6.3% (mol/mol). This amount rose to 64.3 ± 5.8% (mol/mol) after 4 h and was not reduced significantly until 8 h (Figure 13). Compounds with a single hydroxyl group at *C*3 were only detectable in traces. Phloroglucinol, generated from the A-ring yields 15.4 ± 2.1% (mol/mol) of the applied flavan-3-ol within 60 min. The maximum of 35.4 ± 3.3% (mol/mol) was reached after 2 h, thereafter degradation of this intermediary metabolite prevailed over generation. A comparative degradation study of (+)-catechin, (-)-catechin and DL-catechin did not show any quantitative or qualitative differences. Even (-)-epicatechin was degraded equally apart from some small interindividual differences between the donor organisms. This reflects the *in vivo* situation very well (*43*). The results also show, that the complex mixture of microorganisms in the microbiota does not depend on stereochemical aspects, what brings out on the one hand its high hydrolytic abilities. On the other hand the importance of degradation experiments with physiologically composed microorganisms compared to single species studies are emphasized (*8*, *72–74*).

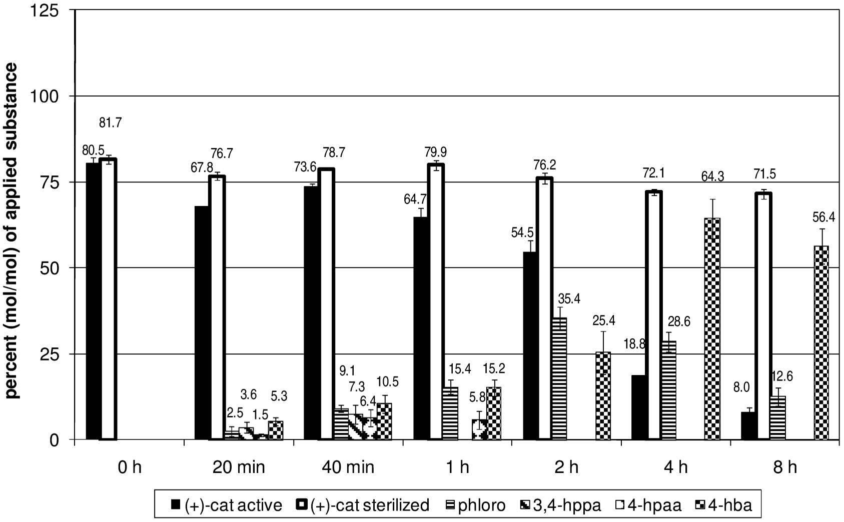


Figure 13. Relative proportions (% (mol/mol)) of (+)-catechin ((+)-cat active) and its degradation products phloroglucinol (phloro), 3,4-dihydroxyphenylpropionic acid (3,4-hppa), 4-hydroxyphenylacetic acid (4-hpaa) and 4-hydroxybenzoic acid (4-hba) in active cecal suspension in reference to (+)-catechin ((+)-cat sterilized) in sterilized inoculum. (n = 6, mean ± SD; measured by HPLC-UVD/-FLD and GC-MS). (43)

If compounds of the gallocatechin type, with an additional hydroxyl group at the C-ring are incubated the pattern of hydroxylated phenylcarboxylic acids is more varying according to higher amounts of 3-hydroxylated isomers (*43*).

Glycosides are unusual for flavan-3-ols, but they can be generated by food processing (*22*, *75*), or occur in some plants like rhubarb or dates (*76–78*). *O*-Glycosides can be found as well as *C*-glycosides. As degradation of *C*-glycosides is less known and the potential bioavailability due to the expected slow microbial metabolism is higher, different catechin-*C*-glycosides have been studied in the pig cecum model. These were amongst others (-)-catechin-8- and -6-*C*-glucoside, as well as -8-*C*-galactoside (Figure 14).

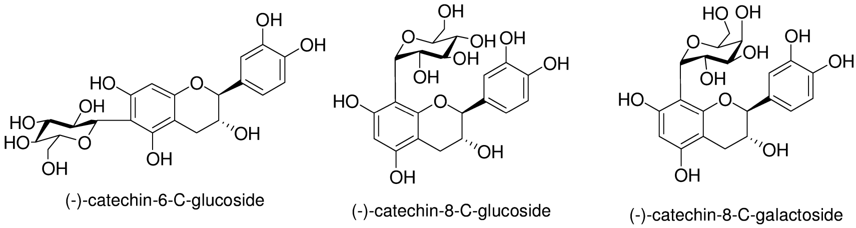

(-)-catechin-6-C-glucoside
(-)-catechin-8-C-glucoside
(-)-catechin-8-C-galactoside

Figure 14. Applied (-)-catechin-C-glycosides.

They are generated during technological step of alkaline treatment of cocoa from (-)-epicatechin and containing sugars by epimerization at *C*2 (Figure 12) and formation of a *C-C* bond via ring opening and rearrangement (*79*). Neither (-)-catechin-8-*C*-glucoside, nor the corresponding galactoside were degraded by the intestinal microbiota within an incubation time of 8 h. The concentration-time course for (-)-catechin-6-*C*-glucoside is shown in Figure 15.

Within 20 min 42.5 ± 0.6% (mol/mol) of the applied compound was degraded. Within the next 40 min additional 6% (mol/mol) were metabolized. At a timepoint between 2 and 4 h (-)-catechin-6-*C*-glucoside was totally degraded. These reactions liberated phloroglucinol as residue of the A-ring with altering amounts of 7.3 ± 1.3% (mol/mol) at 40 min and 23.8 ± 2.4% (mol/mol) after 4 h. On the other hand 3,4-dihydroxyphenylpropionic acid was generated. This intermediary metabolite rose to a concentration of 9.3 ± 1.4 µM after an incubation time of 60 min. The compound was degraded via shortening of the aliphatic chain and dehydroxylation leading mainly to 4-hydroxyphenylacetic acid and 4-hydroxybenzoic acid afterwards (*80*).

The results of the incubation of (-)-catechin-6-*C*-glucoside were in the same magnitude as for flavan-3-ol aglyca which confirms findings of the comparison of apigenin-8-*C*-glycoside (vitexin) and apigenin (*81*). It also confirms results from degradation studies with human colostomy fluid (*79*). Also the finding of non-degraded 8-*C*-glycosides supports different authors: Mangiferin (norathyriol-6-*C*-glucoside) was degraded in a study using human stool samples as source of intestinal microorganisms (*82*), while puerarin (daidzein-8-*C*-glucoside) was also not degraded by microorganisms from human stool samples or in rats (*69*, *83*).

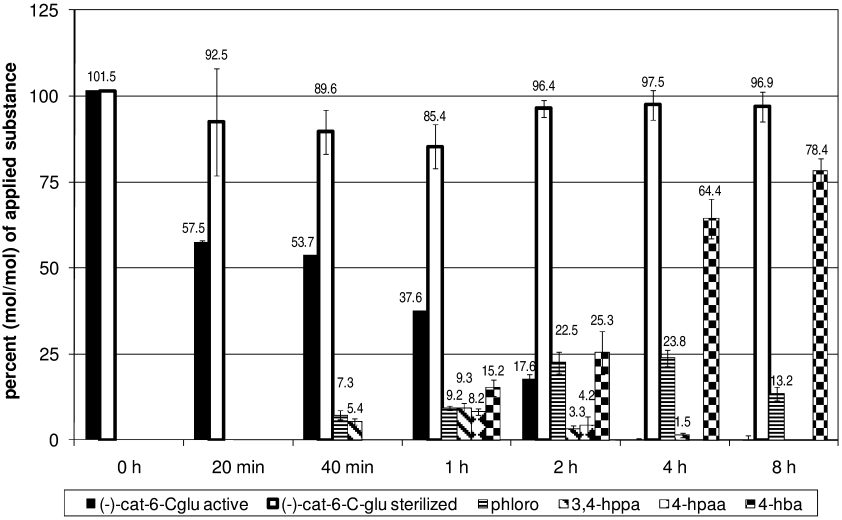


Figure 15. Relative proportions (% (mol/mol)) of (-)-catechin-8-C-glucoside ((-)-cat-6-Cglu active) and its degradation products phloroglucinol (phloro), 3,4-dihydroxyphenylpropionic acid (3,4-hppa), 4-hydroxyphenylacetic acid (4-hpaa) and 4-hydroxybenzoic acid (4-hba) in active cecal suspension in reference to (-)-catechin-8-C-glucoside ((-)-cat-6-Cglu sterilized) in sterilized inoculum. (n = 6, mean ± SD; measured by HPLC-UVD/-FLD and GC-MS). (80)

Degradation of Procyanidins

Procyanidins are oligomers build up from flavan-3-ol monomers. Therefore they do occur in the same plants and foodstuff as flavan-3-ols, but the amount is not easy to determine, what leads to unclear information about daily intake (*70*, *71*). Also the fate of procyanidins in the gastrointestinal tract according degradation and absorption is despite several studies with model systems, animal or human studies (*24*, *25*, *34*, *84–86*) still unclear.

The incubation of procyanidin B2 yielded as an important result the high interindividual differences, which did not allow a reasonable combination of the data for all 3 donor organisms. It can therefore be reasoned, that the results depicted in Figure 16 are analog for humans, because the differences in the chosen model do only reflect the *in vivo*-situation in humans.

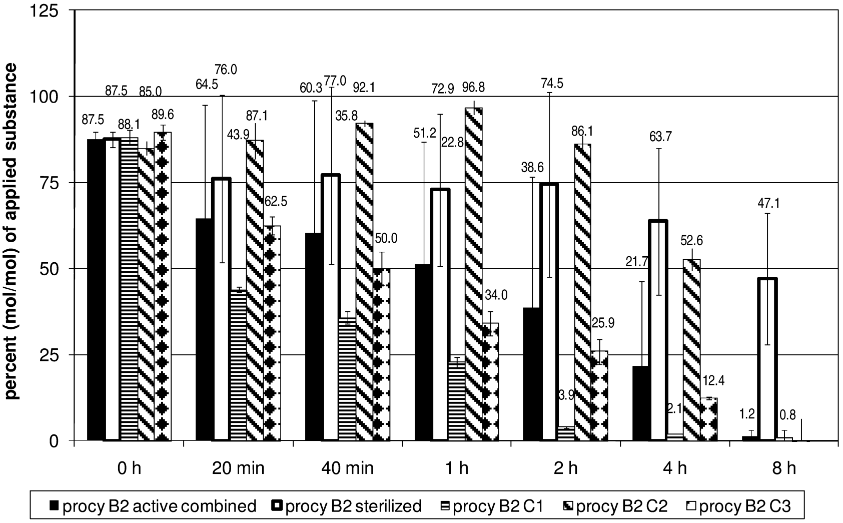


Figure 16. Relative proportions (% (mol/mol)) of procyanidin B2 in active cecal suspension (procy B2 active combined (n=6)) in reference to that in sterilized inoculum (procy B2 sterilized (n = 6)) and the single incubation experiments (procy B2 C1 - 3) (mean ± SD; measured by HPLC-UVD/-FLD). (43)

Within 2 h 61.4 ± 38.1% (mol/mol) of procyanidin B2 are degraded. Afterwards the degradation rate lowers. The applied compound disappears during the whole incubation period of 8 h. Among the 3 different ceca large quantitative differences with regard to the microbial degradation of the dimer are observed. Two donor individuals (Figure 16, procy B2 C1 and procy B2 C3) had a microbiota able to degrade procyanidin B2 directly and nearly total within 4 h, but also with remarkable differences between each other. The microbiota of the third cecum (Figure 16, procy B2 C2) degraded procyanidin B2 much slower up to a timepoint of 4 h, after which 52.6 ± 3.3% (mol/mol) were still available. All control compounds were degraded with a small standard deviation, therefore the collected data clearly show the influence of different sources of the microbiota. Degradation of procyanidin B2 did not proceed via cleavage into monomers, which could not be detected at any timepoint. This is in accordance with DONOVAN *et al.* who found no monomers studying the metabolism of procyanidin B3 and a grapeseed extract containing several procyanidins (*34*). Again an incubation of a mixture of flavonoids resulted in the finding that more complex compounds bearing *C-C* bonds between their main moieties resist microbial degradation longer (*43*).

Concluding Remarks

During the study of intestinal metabolism of flavonoids with the pig cecum model the following findings were remarkable. Deglycosylation of flavonoids by microorganisms of the gastrointestinal tract depends mainly on the type of the sugar and its bond to the aglycone. The intestinal microbiota has a high hydrolytic potential, as nearly all applied compound are degraded at 60% (mol/mol) after an incubation of 2 to 4 h. Anthocyanidins are unstable at near neutral physiological conditions in the colon, but at least they are also degraded via microbial C-ring cleavage. Like other flavonoids these yield phloroglucinol-(-aldehyde) and hydroxylated phenylcarboxylic acids which underlie ongoing bacterial metabolism.

The uptake of compounds through the intestinal barrier is a determining factor for the overall bioavailability of each food constituent and therefore a possible systemic effect. The inner compartment of the mucosa cells can be reached by passive diffusion if aglycones are lipophilic enough (*87*). An active transport of *O*-glycosides by sodium-dependent glucose transporter 1 has been vividly discussed between 1995 and today (*29*, *30*, *88–91*). But this is still as questionable as the necessity of cytosolic β-glucosidase (*27*, *28*, *92*), the influence of enzymes of the brush border membrane (*93–95*), or the mechanism how *C*-glycosides could be transported.

The microbial metabolites of the C-ring cleavage of liberated aglycones, are phloroglucinol(-aldehyde) or different hydroxylated phenylcarboxylic acids. The former is known to be metabolized further to short chain fatty acids (*96*, *97*). Because of this generated metabolites are substrates of the monocarboxylic acid transporter. Even the phenylvalerolactones sometimes named as key metabolites (*84*, *98*, *99*) can be taken up by them. So it can be summarized, that the bacterial metabolites are absorbed in the colon and can reach high plasma concentration which may also have physiological effects (*100*), which is unclear for intact flavonoids.

Another yet unclear point is the bioavailability of complex or bound flavonoids and the role microbiota might play therefore. Food processing is known to influence the content of monomers and dimers in grapes, berries or sorghum (*101*, *102*). But microbial enzymes show also the property to release polyphenolic substances bound to cell wall-material (*103*, *104*) or proteins (*43*). Furthermore microorganisms from the gastrointestinal tract are able to reduce the mean degree of polymerization (*105*). On the one hand the intestinal microbiota degrades flavonoids contained in food, on the other hand it might enhance the amount of available ones. In every case the microbiota influences bioavailability of food constituents enormously and the pig cecum model is one tool to study its mechanisms.

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Chapter 28

Cellular Antioxidant Defenses and Amelioration by Biopigments with Particular Focus on mRNA Oxidations

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All biological molecules are sensitive to oxidation by free radicals, making every living aerobic organism subjective to "oxygen paradox". The dangerous structure of oxygen arises from its electron imbalance leading to its univalent reduction, which in turn generates reactive intermediates in a living cell. The excessive accumulation of such reactive oxygen species (ROS) leads to oxidative stress implicated in a number of degenerative processes such as mutagenesis, cancer, atherosclerosis, congestive heart diseases, chronic inflammatory diseases, macular degeneration, cataract, disorders of central nervous system such as Alzheimer's dementia, Parkinson's disease, including the aging process. A large number of studies on effects of pro-oxidants mainly concern DNA, proteins and lipids. Whereas RNAs, which are distributed throughout the cell in a less protected form than nuclear DNAs, are thus exposed to higher risks of oxidative damage. Since the primary cellular defense is handled by antioxidant enzymes, the ROS-induced damage caused to their mRNAs weakens the cell's defensive strength. This review compiles research developments made towards understanding the oxidative damage caused to nucleic acids, particularly to RNAs and the major biochemical events through which the intrinsic and extrinsic antioxidants function

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towards maintaining normal homeostasis. Finally, the role of carotenoids (lipophilic) and anthocyanins (hydrophilic) bio-pigments in accentuating protection to cells, including to that of mRNAs of antioxidant enzymes is discussed.

Introduction

All eukaryotes contain DNA – the polymer molecule responsible for storing the basic genetic information. During cell multiplication, DNA is duplicated whereas during cell functioning, specific portions of DNA are transcribed into RNA molecules. The latter are then translated into proteins, which in turn regulate all other cellular biochemical processes. It is well understood that the DNA polymer is intensely twisted and protected with histone proteins, together resulting in the formation of nucleosomes which are collectively enveloped by nuclear membrane, thus separating the cellular fluid from the basic genetic material. Although cytoplasmic organelles also carry some DNA, the major portion of the cellular DNA lies within the nucleus, in the form of chromosomes. For all higher organisms, oxygen is needed for supplying the energy to carry out various biochemical processes. Even though oxidation is a requisite for all biological functions, the same oxygen in various forms can be reactive, and hence toxic to living cells. Oxygen atom normally has paired electrons, whereas a ‘reactive oxygen’ atom has an unpaired electron in its outer valence shell and its molecular oxygen (O_2) has two unpaired electrons, thus making the molecule a free radical (also referred as ‘reactive oxygen species’ - ROS). In the process of regaining the chemical stability, such reactive molecules absorb electrons from surrounding molecules, thus causing a series of reactive molecules (*1*). Therefore, the radical nature of molecular oxygen contributes to certain important oxidation/reduction chemistry. The reductive environment of the cellular milieu provides ample opportunities for oxygen to undergo unscheduled univalent reduction thus generating reactive intermediates such as superoxide anion radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH^{\bullet}$). Other oxidants relevant to human pathology are hypochlorous acid (HOCl), peroxynitrite (OONO), reactive aldehydes, lipid peroxides, lipid radicals and nitrogen oxides (*2*). The surplus reactive species formed in living cells leads to a biochemical stress, generally known as ‘oxidative stress’. Under physiological conditions, the major endogenous source of oxygen radicals is the mitochondrial electron transport chain, generating chiefly the superoxide anions (*3*, *4*). Other sources of oxygen radical generation are the phagocytic cells such as neutrophils, monocytes and macrophages during phagocytosis and auto-oxidation of biological molecules (*5*–*7*). The exogenous sources of free radicals are radiation, smoke, environmental pollutants, detergents, ozone, various toxicants, sonication and other physical agents.

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Oxidation and Oxidative Stress

Despite being a relatively young field, the study of oxidative stress has attracted huge interest. A growing number of groups have been able to assess stress-generated pro-oxidant biomolecules causing an array of pathologies in mammalian cells. As a response, cells trigger redox-sensitive regulatory molecules to inhibit the cellular injury (*8*).

In all higher organisms, the cells are equipped with the ability to overcome the ravaging effects of the free radicals by generating an assortment of hydrophilic and lipophilic antioxidant molecules and antioxidant enzymes (AOE), and also through other antioxidant molecules assimilated through diet. Oxidative stress generally results in an increased AOE activity to prepare the cell against oxidative injury; however, the increased activity is not always associated with an increased gene expression and vice versa (*9–13*). Under normal physiological conditions, a homeostasis exists between the reactive species and the antioxidant defense. When the levels of reactive species surpass the cell's antioxidant capacity, as does in a pathological state, this homeostasis is altered and oxidative stress ensues. In recent years these reactive species have been implicated in a wide range of degenerative processes and diseases such as mutagenesis (*14*), cell transformation, cancer, atherosclerosis, arteriosclerosis (*2*, *15*), cardiovascular disorders (*16*), chronic inflammatory diseases such as rheumatoid arthritis, lupus erythematosus and psoriatic arthritis; acute inflammatory problems such as wound healing (*17–19*); photo-oxidative stresses to the eye such as cataract, central nervous system disorders such as Alzeimer's dementia, Parkinson's disease (*20*, *21*) and a wide variety of age-related disorders and the aging process itself (*22*, *23*). Because free radicals are implicated in all these processes (*23*), minimizing and neutralizing their activity with antioxidants may allow humans to live longer and healthier, look and feel better, and reduce or eliminate the risk of certain physiological disorders and diseases. Some of these oxidation-linked diseases and syndromes can be aggravated, perhaps even initiated, by various pro-oxidants. Pro-oxidants can be either biologically, biochemically and physiologically generated or derived from the environment.

Oxidation of RNA

RNA oxidations, as those of DNA, are expected to cause more deleterious effects on cellular functions, because of the crucial and decisive roles played by them. However, a large number of studies focus on the oxidative damage caused to cellular biomolecules rather than the nucleic acids. Between the two major functional nucleic acids, a much higher number of reports focus on DNA damage than RNA damage, although the latter (RNA) is equally functional and less protected than DNA. **Box-1** summarizes the major differences between DNA and RNA, which indicate higher chances for RNA, rather than DNA, to undergo oxidative damage. Considering that there is generally more RNA in a cell than DNA, it is likely that there will be a significant damage to cellular RNA when cells are exposed to cytotoxic substances (*23*, *24*). Factors that cause damage to DNA would also damage RNA, due to their structural similarity. In fact, RNA is

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more amenable to certain types of free radical attack than is DNA, owing to (i) its widespread cytosolic distribution (ii) its single stranded structure in many areas of the molecule (iii) the absence of protective histones (iv) the absence of advanced repair mechanisms and (v) the presence of free oxygen group. While a higher damage to RNA than to DNA has been demonstrated both in *vivo* and *in vitro* (*25*), any damage occurring in an mRNA molecule may result in abnormal protein translation whereas tRNA and rRNA damage could result in dysfunction of protein synthesis (*15*, *23*). The amount of oxidative damage to nucleic acids was elevated in the CA1, CA3 and dentate gyrus regions of the hippocampus among patients with bipolar disorder, schizophrenia and major depressive disorder. This damage was predominantly in the cytoplasm, suggesting that the damage was primarily to RNA. Compared with oxidative damage in control samples, the magnitude of damage was high in patients with schizophrenia, modest in patients with bipolar disorder and lower in patients with major depression. Oxidative damage to RNA but not DNA was observed in the hippocampus of patients with major mental illness (*23*, *26–29*).

mRNAs of Antioxidant Enzymes

Several studies have demonstrated that, like most of the RNAs, the levels of mRNAs coding for AOEs such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) as well as their activities are altered in conditions of diseases and stress, causing further aggravation of the biological stress. Exposure of cultured cells and in vivo models to agents that lead to oxidative stress also cause an increase in levels of mRNAs of certain AOEs, indicating their over-expression is often indicative of their role in combating oxidative stress. There are also reports that mRNA oxidation is an early event in causing cell death (*30*). Thus, studying the status/quantum of relevant mRNA can reveal damage/protection rendered by pro-oxidative or antioxidative molecules in food. Healthy and active cells are generally well equipped to cope with normally encountered levels of reactive species with its antioxidant enzymes and glutathione redox system. Several studies have observed that the presence of small, continuous stimulus such as ROS induced the expression of antioxidant enzymes as a mechanism of defense (*31*). Exposure of human endothelial cells to hyperoxia increased the mRNA levels of CAT, GPx and SOD (*32*). Thus, tracking changes in activities of endogenous antioxidant enzymes and their gene expression levels is considered a fairly sensitive biomarker of the cellular oxidative stress.

A large number of genes have evolved in eukaryotes with the rationale of providing protection to cells from the toxic effects of xenobiotics and other toxic chemicals. Therefore it is imperative to understand the roles of these genes in determining the cellular responses to oxidative stress. A number of biochemical models have been developed over a period of several decades including that of enzyme knock-out (or null) models (*33*, *34*), which play a pivotal role in establishing the role of oxidants and antioxidants and other drug molecules in maintenance of health and alleviation of disease states.

Apart from transcription, a change in an antioxidant enzyme activity may also arise from translational regulation of gene expression. A study on the effect of H_2O_2 and paraquat on the antioxidant status of rat astroglial cells revealed a transcriptional regulation of MnSOD and a post transcriptional regulation of CAT gene expression (*35*). The transcription of antioxidant enzymes is controlled by certain transcriptional regulators which are activated in response to oxidative stress. Mouse GPx and CAT genes possess putative binding motifs for the transcriptional regulators NF-κB and AP-1, which are involved in the up regulation of GPx and CAT in response to oxidative stress (*36*). Two characteristic regulatory elements called, the antioxidant responsive element (ARE) and xenobiotics responsive element (XRE), present on the promoter region of the CuZnSOD gene of human liver cells induce up regulation of this gene under oxidative stress (*37*). Thus the antioxidant enzymes can be transcriptionally induced by the above transcription factors in combination with or independently of these two regulatory elements to form a defense against oxidative stress (*38*) while a posttranscriptional regulation cannot be ruled out. Shull et al (1991) observed that the antioxidant enzymes are selectively induced when exposed to different ROS generating systems. For example, exposure of tracheobronchial epithelial cells *in vitro* to H_2O_2 induced a significant elevation in the steady state mRNA expression of CAT while that of MnSOD and GPx remained unaltered. However, an exposure to xanthine/xanthine oxidase system induced the expression of MnSOD with no effect on the other antioxidant enzymes (*9*). Therefore, an understanding of the different regulatory elements involved in gene expression in oxidative stress conditions and their reversal by antioxidants is important in developing antioxidant therapy.

While mRNAs for various proteins are transcribed and continuously released into the cytoplasm, they are available for translation for a specific period. Therefore, their stability is very important which acts as an index of the turnover of these enzymes. Extension of half-life invariably is an indication of ensuring mRNA availability for translation. Thus, delay of mRNA decay via repair mechanisms offered by exogenous antioxidant such as pigments, flavonoids and terpenoids ingested through food would unravel the latter's efficacy in conferring protection for RNA. A recent report suggests that anthocyanins induce phase II antioxidant enzymes through activation of antioxidant response element (ARE) upstream of antioxidant genes (*39*). Dietary supplementation with β-carotene significantly enhanced the activity of GPx and moderately enhanced the activity of SOD in rats with ischemic reperfusion injury (*40*). Difference in longevity between males and females is correlated to the lower ROS and higher expression of antioxidant enzymes in females which in turn is attributed to elevated estrogen level, which up regulates antioxidant expression through activation of estrogen responsive elements (*41*). Over-expression of antioxidant enzymes is associated with an increase in life span. Up regulation of SOD and CAT in transgenic *Drosophila melanogaster* increased the lifespan by about 50% (*42*, *43*) which is again higher in females (*44*). The finding by Schriner et al (*45*) revealed an increase in murine life span by targeted over-expression of mitochondrial CAT with additional benefits of delayed cardiac pathology and cataract, reduced oxidative damage and mitochondrial deletions. Therefore, targeting such specific

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sites to over express antioxidant genes by dietary antioxidant intervention may indeed help extend a healthy life span in humans.

Stability of mRNAs

Modulation of gene expression at the level of mRNA stability has emerged as an important regulatory paradigm. Concentration of an mRNA is a function of its rate of synthesis and rate of degradation. Therefore, the half-life of mRNA in the cell is an important determinant of gene expression and its translation into the protein product (*46*).

Transcriptional regulation of antioxidant enzymes is well known. CAT gene expression in mouse skeletal muscles is transcriptionally regulated (*47*), which is associated with NF-κB and AP-1 factors (*36*). However, post transcriptional regulation is becoming evident especially through stabilization of the mRNA transcripts. For example, rise in CAT mRNA levels after exposure to low levels of H_2O_2 is found to be post transcriptionally regulated through increased stabilization of the same in V79 fibroblasts (*48*). Similarly, enhanced expression of CAT gene in hyperoxia-exposed rat lung is linked to the increased CAT mRNA stability (*49*). Therefore, a change in the abundance of a particular RNA may reflect a change in the transcriptional activity of the gene, a change in the rate of turnover of the specific RNA or both. The rate of degradation of a specific RNA molecule can be measured by blocking the synthesis of all RNA molecules. Thus stability (against oxidation) of a batch of mRNA, or even total RNAs can be measured by blocking its synthesis with a transcription inhibitor, isolating cytoplasmic RNA at different time intervals and monitoring the rate of loss of a particular transcript with a gene specific probe. The most commonly used transcription inhibitors are actinomycin D, α-amanitin, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole and cordycepin (*46*). However each inhibitor has its own disadvantages in that α-amanitin does not enter all cells while actinomycin D also inhibits translation and affects ATP pools. Also, the results obtained with different methods differ considerably. Stability of mRNA molecules along with transcription, mRNA processing, translational efficiency and post translational modification is an important determinant of protein levels in the cells.

Increased stability of mRNA transcripts generally means a steady state translation into the protein product. This property is desirable in antioxidant therapy especially in cases where an oxidative stress results in reduction of antioxidant defense. Drugs that increase the stability of antioxidant enzyme transcripts are especially advantageous in order to increase the resistance towards oxidative insult. While most studies focusing on the effects of dietary antioxidants are limited to antioxidant enzymes and their egulations, study on their effects on stability of the respective mRNA transcripts is lacking. Since natural plant extracts which are known to impart antioxidant effects may also enhance the stability of mRNAs of CAT, SOD and GPx against oxidative stress, studies need to be conducted in this direction. Our experiments have shown that anthocyanin pigments from the Black Java plum berries efficiently reversed TBH-induced oxidative damage to the mRNAs of antioxidant enzymes(unpublished).

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Oxidation of DNA

Reactive oxygen species react not only with lipids and proteins but also with nucleic acids, thereby inducing oxidative damage to DNA and RNA. Guanine in DNA and RNA is more sensitive to ROS attacks than are the other bases and ROS oxidize guanine and generate 8-oxo-7,8-dihydroguanosine (8-OHG) in RNA and 8-oxo-7,8-dihydro-2-deoxyguanosine (8-OHdG) in DNA (*27*).ROS such as $O_2^{-\bullet}$, H_2O_2, $OH^\bullet$ can damage DNA leading to single strand breaks, double strand breaks or chromosomal aberrations (*1*) that is manifested in mutagenesis, carcinogenesis, and aging. The chief sources of ROS species that account for much of the DNA damage in biological system are the Fenton system, ionizing radiation and nuclease activation (*50*) through the generation of $OH^\bullet$. A number of factors have been identified to cause damage to DNA, mediated through free radical generation. The chief factors include ionizing radiation, transition metal ions, smoke and other chemical carcinogens (*51–53*). Mechanism of damage involve abstractions and addition reactions by free radicals leading to carbon-centered sugar radicals and $OH^\bullet$ or $H^\bullet$ adduct radicals of heterocyclic bases (*54*). Elevated levels of DNA damage have been witnessed in various disease states and it has been hypothesized that such an elevation may be a causative factor in the etiology of such diseases. An active DNA repair system is critical for preventing the occurrence of mutations that may lead to carcinogenesis and other pathologies. Natural products play a pivotal role in offering protection to DNA, thus aiding in the prevention of their base modification or single/double strand breaks. Flavonoids such as naringenin from grape fruit, EGCG from green tea (*55*), quercetin, an almost ubiquitous flavonoid and carotenoids have received much interest in this area due to their proven DNA protecting effects and consequent cancer preventive properties.

Antioxidants

Various studies support the oxidative theory of aging and its delay by manipulation of the antioxidant environment of the organism suggesting the requirement of interventions by antioxidants as the viable target for the prevention or delay of some, if not all, age-related diseases. Antioxidants are substances that reduce, neutralise and prevent the damage done to body by free radicals. They also recover the level of intracellular antioxidants (vitamins, methionine, glutathione and glutathione-related minerals) and are generally required in small concentrations compared to the biomolecules they are supposed to protect, can prevent or reduce the extent of oxidative destruction of biomolecules. There are an infinite number of antioxidants which act by different mechanisms to neutralize the effect of free radicals. These antioxidants may be classified as

- Antioxidant enzymes- catalase the breakdown of free radicals
- Chain-breaking antioxidants- donate or receive an electron to inhibit the free radicals from generating more free radicals
- Metal binding proteins- prevent metal ions from forming free radicals.

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Endogenous Antioxidants

Enzymes

The whole process of free radical generation and their quenching is a concerted action involving a battery of detoxifying catalytic proteins named antioxidant enzymes. The superoxide that is formed during the respiratory chain is converted to H_2O_2 by superoxide dismutase. The H_2O_2, if not removed, interacts with transition metal ions such as Fe^{2+} (Fenton's reaction) producing highly reactive hydroxyl radical. As indicated earlier, endogenous antioxidant enzymes CAT, DOD and GPx comprise a principal system built in all aerobic organisms to defend oxidative stress. Increased activities of these antioxidant enzymes play a key role in increasing the longevity in humans (*56*) by efficiently counteracting the reactive species generated and aid in maintaining the intracellular redox homeostasis. Apart from these, there are other endogenous molecules such as glutathione (GSH)and selenium (Se) supporting the normal functioning of antioxidant enzymes (*57*). Metal binding proteins such as lactoferrin, transferrin (iron-binding proteins) and ceruloplasmin (Cu-binding protein) bind to transition metal ions that otherwise participate in the formation of reactive species (*58*), thus playing an important role in the amelioration of pathological states (*59–61*).

It is a general consensus that oxidative stress results in an increased activity of AOEs such as catalase, glutathione peroxidase and superoxide dismutase. Shull et al (*9*) reported a selective AOE inductive response of tracheobronchial epithelial cells to different oxidative insults. The AOE response depends on the toxin either up-regulating or down-regulating the same. For instance, while toxins like CCl_4, alcohol, paraquat and microcystine, a microbial toxin lower the cells' AOE activities (*62–64*), others like TBH and rotenone increase the activity of the enzyme in response to the stress (*38*, *65*). However, stress response of a particular cell or tissue type may vary with the ensuing pathology or the type of oxidative stress. For example, activity of the AOEs varied with tissue type in streptozotocin induced diabetic rats. While the activities of specific AOEs were up regulated in certain tissues, they were down regulated in others (*66*) suggesting that the degree of stress response is perhaps tissue specific. The activities of AOEs were found to decrease in CCl_4-exposed isolated rat hepatocytes as described earlier in this chapter. Further studies were carried out to elucidate the cellular response to yet another oxidant, TBH in terms of AOE activity and their gene expression and the same is discussed in the following sections.

The measurement of changes in endogenous antioxidant enzyme activity is considered a fairly sensitive biomarker of the response to oxidative stress. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are primary antioxidant enzymes that protect cells from damage caused by ROS.

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Superoxide Dismutase (SOD)

Superoxide dismutase is the primary defense against damage caused by $O_2^{\bullet-}$ and its reactive progeny. Superoxide radicals generated by the respiratory chain through the reduction of molecular oxygen are highly damaging to the cells. Superoxide radical is also formed by the univalent reduction of oxygen during various enzymatic reactions and leukocyte phagocytosis. However, within the cell, superoxide radicals produced are physiolo gically inactivated by the mitochondrial superoxide dismutase to molecular oxygen and H_2O_2 which is further inactivated by the H_2O_2–inactivating enzymes (*67*, *68*).

Three forms of SODs have been identified and characterized in humans: cytosolic Cu-Zn SOD, mitochondrial Mn SOD and extracellular EC SOD. Cu-Zn SOD is the major intracellular SOD and is a dimeric protein (32kDa) with two identical subunits. Each subunit contains a Zn atom and a Cu atom. Zinc atom helps stabilize the protein while Cu is responsible for the activity of the enzyme that is, conversion of two superoxide molecules into H_2O_2 and H_2O (*69*) displaying a diffusion limited pseudo first order reaction kinetics ($k= 3x10^9\ M^{-1}\ s^{-1}$). It is present in the cytoplasm and nucleus where it mainly functions as a superoxide scavenger (*70*). The loss or dysfunction of either Cu-Zn SOD or Mn SOD has been associated with ROS-mediated pathologies. For example, mutated Cu-Zn SOD proteins have been linked to instances of amyotrophic lateral sclerosis (*71*) while loss of Mn SOD has been associated with neonatal death. Postnatal exposure to ambient oxygen results in increased susceptibility to mitochondrial oxidative injury of neurons of the central nervous system, cardiac myocytes and other metabolically active sites in Mn SOD deficient mice (*72*). Liochev and Fridovich (*73*, *74*) proposed that an excess SOD should decrease the steady-state level of $O_2^{\bullet-}$ with no subsequent increase in the endogenous H_2O_2 formation. This was supported by Teixeira et al (*75*), where cell variants over-expressing Cu-Zn SOD were shown to exhibit low steady state levels of H_2O_2, even under conditions of oxidative stress. They demonstrated that the reduction in steady state levels of H_2O_2 is in fact, a direct effect of reduction of $O_2^{\bullet-}$ due to the higher cytosolic activity of the enzyme aconitase. Several natural products cause enhanced activities of antioxidant enzymes in higher animals. Modest induction of SOD has been found to be associated with reduction in the age-related increase of lipid peroxidation products in human plasma (*76*). Transgenic over-expression of SOD and CAT in *Drosophila melanogaster* significantly increased its life span (*43*). Over-expression of SOD in mammals has been found to be linked with increase in longevity (*77*, *78*). Nelson et al (*76*), proposed a fundamentally different approach of safely and modestly inducing the antioxidant enzymes *in vivo* by administration of antioxidant supplements in moderate doses. This may prove to be a powerful tool in studying oxidative stress and diseases associated therein.

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Glutathione Peroxidase (GPx)

Glutathione peroxidases (GPxes) are a group of selenocysteine containing proteins playing a central role in the reduction of H_2O_2 and a wide range of organic hydroperoxides to water and their corresponding alcohols respectively (*79*, *80*) and acts as peroxynitrite reductase (*81*). The catalytic reactions effected by GPx are shown below:

$$H_2O_2 + 2\ GSH \xrightarrow{GPx} 2\ H_2O + GSSG$$

$$ROOH + 2\ GSH \xrightarrow{GPx} ROH + H_2O + GSSG$$

The glutathione is regenerated from the GSSG by the enzyme glutathione reductase. The catalytic site in GPx is identified to be the selenocysteine moiety (*82*). Depending on their cellular location and substrate specificities, four isozymes of GPx are recognized in higher organisms viz., cellular (GPx1), gastrointestinal (GPx2), extracellular or plasma (GPx3) and phospholipid (GPx4) glutathione peroxide. Among the four, GPx1 is the most abundant. Mills (*79*), for the first time showed the presence and action of GPx in erythrocytes, where it was found to inhibit the oxidative breakdown of hemoglobin in the presence of H_2O_2. Ever since then this family of enzymes has drawn attention of many researchers pertaining to its importance in health and diseases. Gpx activity is primarily dependent on Se. Selenium deficiency is directly associated with decreased enzyme activity and GPx protein (*83*) as well as its cytoplasmic mRNA abundance (*84*). The proof for its protective effects against oxidative stress is innumerable. The knock-out mice which lack GPx1 are sensitive to oxidative stress by xenobiotics such as diquat, paraquat, H_2O_2etc and eventually die of excess oxidative damage (*34*, *85*, *86*). About 30% reduction in GPx activity was observed when rat hepatocytes were challenged with CCl_4 as compared with the untreated cells (*87*).

There have been contradictory reports on activity of GPx in cells due to the effect of the other pro-oxidant, TBH. While Ochi et al, (*88*) report the inhibitory effect on GPx, there are others who claim an opposite effect (*38*, *89–91*). It is possible that this difference is due to the difference in the type of cell line they used in their individual experiments. This enzyme was found to be inhibited by 50μM of TBH in vitro (*92*). However, this inhibition is perhaps due to the direct effect of the organic hydroperoxide on the purified enzyme in vitro. A direct contact of the protein with TBH seldom occur in vivo, due to the presence of biological barriers and other cellular factors which might protect the protein against the inhibitory activities of the oxidant. Another theory suggests that the induction of molecular homolysis of hydroperoxides by unsaturated fatty acids of membrane lipids leads to lipid auto-oxidation producing lipid hydroperoxides resulting in more free radicals. Therefore, the free radicals derived from such processes or some consequent non-radical products may serve as inducers of

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the glutathione enzyme system rather than the hydroperoxide substrates (*93*). Therefore, what exactly is the reason for the difference in GPx activity in different cell types under TBH stress is still unknown.

Regarding the mechanism, the activity of GPx1 is regulated by a signaling pathway that is activated in response to oxidative stress. The c-Abl and Arg SH3 domains of the respective tyrosine kinases bind to the proline-rich sites on GPx1, thus stimulating its activity and this association is controlled by the cell's oxidant level (*94*). GPx is particularly important in those cells, whose mitochondria lack the CAT, where it does most of the detoxification of H_2O_2 (*95*). The protective role of GPx1 has been unequivocally established in various models (particularly in vivo) such as ischemia/reperfusion injury (*96*), virus-induced myocarditis (*97*), endotoxemia (*98*), diabetes-associated atherosclerosis (*99*) and pro-oxidant induced neurotoxicity (*100*). Sies et al (*81*) demonstrated its GSH dependent reduction of peroxynitrite to nitrite which attenuates the peroxynitrite mediated protein nitration in human fibroblast lysates. This might have physiological relevance in inhibiting the peroxynitrite induced apoptosis in cells. They later demonstrated that in vivo, GPx acts differently by actually accentuating peroxynitrite-induced apoptosis which is perhaps due to the ineffective enzymatic reduction of peroxynitrite in culture where it encounters more reactive peroxynitrite intermediates such as $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ (*101*). Therefore, it appears that the activity of GPx mainly depends on the type of oxidant and hence the judicial supplementation of antioxidant must be considered, keeping in mind the implications of such compounds under different stress conditions.

The phospholipid hydroperoxideGPx is considered to be involved in protection of lipid biomembranes against oxidative peroxidation (*102*). The increased reduction in lipid peroxidation by the extracts and standards appears to be the result of increased action of GPx thus conferring enhanced protection against TBH induced lipid peroxidation. Natural products, particularly the green tea is reported to increase GPx activity in livers of alcohol intoxicated aged rats. It significantly increased the GPx activity in rat liver as compared to vehicle treated, control rats (*103*), which also could be by way of inhibiting the oxidative damage to their mRNAs and their stability. EGCG being the chief constituent of green tea may be partly responsible for increased GPx activity. Similarly, the flavonoids and other polyphenols have been credited with the ability to enhance antioxidant capacity of living cells (*104*, *105*) either by reducing the burden on the endogenous AOEs or by imparting protection to their mRNA damage. Despite the contradictory reports on the regulation of AOE activities, the polyphenols appear to confer protection against oxidative stress perhaps via mechanisms independent of or in combination with other pathways. The results of Alia et al (*106*), where quercetin alone did not induce a significant change in the activity of GPx but increased the mRNA expression significantly in hepatoma cells at similar concentration tested in our study. Similarly, a reduction of TBH-induced increase in GPx activity was reported by quercetin (*107*) which is again in contrast to the current study. Rohrdanz et al (*108*) demonstrated a decrease in mRNA expression of GPx by quercetin. Anthocyanins have been shown to increase the mRNA expression of AOEs in different cell and animal models (*109*, *110*). It is reported elsewhere that EGCG even at µM concentrations showed oxidative stress enhancing effects as evidenced by the generation of H_2O_2 (*111*). They observed that the toxic effect of EGCG was significantly higher than that of the generated H_2O_2 amounts added exogenously suggesting that the toxic effects of EGCG cannot be fully ascribed to H_2O_2 generation but may be due to the formation of superoxide. A very high activity of SOD and its expression may be correlated with the toxicity. However, in light of several other reports which demonstrate the antioxidant effects of EGCG (*103*, *112*), particularly at low concentrations, such a claim has to be viewed with care and much deliberation.

Catalase (CAT)

This enzyme is a homotetrameric heme containing protein which decomposes H_2O_2 to molecular oxygen and water in tandem with GPx. Catalase can decompose H_2O_2 catalysed by two different modes of enzymatic activity: the catalytic mode of activity (reaction 1) and the peroxidative mode of activity (reaction 2)

$$2\,H_2O_2 \xrightarrow{CAT} H_2O + O_2 \qquad (1)$$

$$H_2O_2 + AH_2 \xrightarrow{CAT} A + 2\,H_2O \qquad (2)$$

The role of CAT in defending cells and tissues against oxidative stress has been studied extensively. Over-expression of CAT renders cells more resistant to toxicity of H_2O_2 and oxidant-induced injury from exposure to hyperoxia (*113*, *114*). Most cells lack CAT in their mitochondrial. In such cells, mitochondrial superoxide anion and hydrogen peroxide are dismutated by SOD and GPx and the significant amount of H_2O_2 that diffuses into the cytosol, despite the action of SOD and GPx in mitochondria, is detoxified by the cytosolic CAT. Since mitochondria are the main sources of ROS, a consequence of oxidative phosphorylation, they are highly susceptible to oxidative damage. Mitochondrial over-expression of CAT has been found to provide better protection than cytosolic over-expression against H_2O_2 induced lung injury (*115*). It is also shown to increase the median life span in experimental animals associated with delayed onset of cardiac lesions and cataract development and reduction in oxidative damage, H_2O_2 production, H_2O_2-induced aconitase inactivation and mitochondrial DNA lesions (*45*). Catalase knock-out mice show normal and healthy signs of development but show varying degrees of sensitivity to oxidative damage depending on the tissue and oxidant type (*116*). Aging is characterized by progressive decline in physiological functioning of multiple organ systems and a steady increase in chronic degenerative diseases. Over-expression of mitochondrial CAT in aging mice is associated with reduction in a number of age-related pathologies such as malignant non-hematopoietic tumor

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burden, reduced cardiac lesions and reduced systemic inflammation. However, it had no effect on hematopoietic neoplasia or glomerulopathy (*117*).

Studies on end-stage failing heart has revealed an up regulation of CAT as a compensatory means for increased oxidative stress (*118*). Treatment with TBH significantly stimulated the activity of the AOEs in isolated rat hepatocytes. The increase in CAT activity in cells treated with EGCG is perhaps a cell's means of defending itself from the harmful effects of H_2O_2. However, at doses above 100 μM, EGCG induces chromosomal damage due to the production of H_2O_2 (*119*). Rohrdanz et al (*35*) demonstrated an increase in CAT-mRNA transcript level when rat brain astrocytes were exposed to H_2O_2 and paraquat. However, Alia et al (*107*) demonstrated a no difference in mRNA expression of CAT in HepG2 cells treated with TBH from control cells despite an increase in the activity under oxidative stress. They also observed a further attenuation of CAT expression in cells treated with quercetin prior to TBH exposure. While TBH had no effect on CAT activity in Chinese hamster V79 cells (*88*), it significantly induced CAT activity in HepG2 cells without affecting the mRNA expression (*107*). In the present study, though CF and XF increased the activity of the enzyme drastically, a parallel increase in mRNA transcript was not observed. Apparently, the activity and expression of AOEs are not inter-related. Conceivably there are transcriptional and/or post transcriptional regulatory mechanisms operating at different levels depending upon the tissue type and/or the oxidant molecule.

Glutathione

Glutathione is a small molecule that plays key regulatory roles in metabolic and cell-cycle-related functions and is responsible for detoxification of ROS by acting as peroxide scavengers and regulating the redox state of cells. This cysteine containing tripeptide (γ-glutamylcysteinyl glycine), which is found in milli molar concentrations in animal cells, provides the principal intracellular defense against oxidative stress and participates in detoxification of xenobiotics aiding in the maintenance of intracellular redox status. Depletion of GSH is one of the chief causes of cell death. For example, overdose of acetaminophen (paracetamol) results in hepatic and renal failure and ultimately in death due to cellular GSH depletion (*120*) indication that it is an essential component of the human immune response. Low intracellular glutathione levels in antigen-presenting cells correlated with defective processing of antigen with disulfide bonds, indicating that this thiol may be a critical factor in regulating productive antigen processing (*121*). Externally supplemented GSH has negligible systemic bioavailability. Therefore synthesis of GSH in conditions of its deficiency is enhanced by means of its precursors such as cystine. Depletion of GSH has been implicated in hepatic and renal injury caused by overdose of drugs like paracetamol (*120*). *Tert*-butyl-hydroperoxide treatment results in rapid depletion of glutathione in hepatocytes (*121*) chiefly via metabolism by GPx and therefore is a suitable model to study glutathione depletion and consequent cytotoxicity in cells (*122*). Natural products have been suggested to enhance in vivo GSH synthesis in a number of cell models by increasing the level of γ-glutamyl-cyseinyl-synthetase, a rate limiting enzyme in GSH synthesis (*123*). Therefore, supplementing with antioxidant biomolecules that stimulate the synthesis of GSH in vivo may be a rational way of overcoming GSH deficiency. Cytotoxicity by TBH has been chiefly attributed to depletion of GSH and increase in phosphorylase-a activity with an associated increase in lipid peroxidation and membrane damage (*124*). Glutathione is crucial in protecting the cells against ravaging effects of reactive species which are reduced by GSH in the presence of GPx resulting in oxidation of GSH to GSSG which in turn is reduced back to GSH by glutathione reductase at the expense of NADPH, thus maintaining an adequate cellular GSH pool.

Protection by Way of Apoptosis

Apoptosis, otherwise known as 'programmed cell death' is a highly regulated innate mechanism in higher animals and humans designed to eliminate highly poisoned cells that cannot be metabologically repaired. The apoptosis process involves a battery of signaling events including caspases -the apoptotic family of proteins and other transcription factors. Oxidative stress, leading to the formation of free radicals is often implicated in ensuing cell death, either apoptotic or necrotic. Necrosis is caused by catastrophic toxic and traumatic events with passive cell swelling, injury to cytoplasmic organelles, rapid collapse of internal homeostasis leading to membrane lysis, release of cellular contents and ultimately resulting in inflammation. On the contrary, apoptosis is a programmed cell death which is characterized by cell shrinkage, membrane blebbing, nuclear fragmentation and chromatin condensation. Thus apoptosis is a highly co-ordinated genetically programmed mechanism involving a cascade of genes which are either up- or down-regulated during the process (*125*). Various experiments indicate that apoptosis can be induced by oxidative stress induced by externally applied xenobiotics such as H_2O_2, organic hydroperoxides-TBH, cumene hydropeorixide etc. (*126*, *127*). Events of apoptosis within a cell can be monitored by microscopic fluorescence imaging using specific fluorochromes such as propidium iodide (PI), acridine orange, ethidium bromide, 4′6-diamidino-2-phenylindole (DAPI), bisbenzimide, annexin V etc., either alone or in combination. While the first three dyes specifically bind to nucleic acids, annexin V is specific to phosphotidyl serine, the molecules that become exposed to the outer plasma membrane leaflet due to the loss of its asymmetry, a hall mark of early apoptosis.

Caspases belong to the family of cysteinyl proteases considered to be executive factors for apoptosis. Estimation of caspase activation and their mRNAs is another method of detection of apoptosis. There are two types of caspases, the initiator caspases (caspase 8 and 9) and the effector caspases (caspase 3 and 6). It has been believed that activation of caspase is induced through two pathways. In the first pathway, binding of cytochrome c released from mitochondria to Apf-1 participates in the activation of caspase-9. In the second pathway, binding of death ligand to death receptors such as Fas and TNF, the latter receptor is known to participate in the activation of caspase-8. Caspase-8 and caspase-9, in turn, activate caspase-3 (*128*, *129*) and the activated caspase-3 cleaves poly (ADP-ribose) polymerase (PARP) (*130*), a hallmark of apoptosis. The final

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outcome of these proteolytic cascades is the specific cleavage of a wide variety of substrates that are implicated in apoptosis.

A number of genes belonging to the Bcl-2 family have been identified which play a pivotal role in apoptotic process. While few members such as Bcl-2, Bcl-xL, Mcl-1 and BAG-1 act as inhibitors of apoptosis, Bad, Bid, Bax, Bak, and Bik act as promoters of apoptosis. Both Bcl-2 (anti-apoptotic) and the Bax (pro-apoptotic) are localized in the outer mitochondrial membranes, nuclear envelope and edoplasmic reticulum (*131*) where ROS are generated and are activated in response to external stimuli through signal transduction process involving several proteins. Some studies have established that the Bcl-2 family of proteins exerts both anti-apoptotic and pro-apoptotic activity through altering the mitochondrial membrane permeability (*132*). The product of Bcl-2gene, the Bcl-2 protein has been localized to mitochondria, endoplasmic reticulum, and the membranes of the nuclear envelope. Bcl-2 is known to regulate mitochondrial membrane potential and be tightly associated with the mitochondrial pore transition, pore regulation, the release of cytochrome c and other apoptosis inducing factors. Bax is a pro-apoptotic protein of the Bcl-2 protein family that resides in the outer mitochondrial membrane. It is controversial whether Bax promotes cell death directly through its putative function as a channel protein or indirectly by inhibiting cellular regulators of the cell death proteases, particularly the caspases. Recent reports have indicated that after exposure to certain apoptotic stimuli, Bax is translocated from the cytoplasm to the mitochondrial membrane and that this process is essential for cell death to occur and found the same results in post-mortem dopaminergic neurons of Parkinson disease (*133*). The organic hydroperoxide TBH is reported to cause apoptosis in immune cells by up regulation of pro-apoptotic proteins Bax and Bid and down regulation of the anti-apoptotic Bcl-2 accompanied by release of cytochrome C (*127*) suggesting a mitochondrial pathway. Anti-apoptotic factors such as Bcl-2 inhibit cell death by stabilizing the mitochondrial membrane, while pro-apoptotic ones such as Bax and Bid induce cell death by increasing the mitochondrial membrane permeability leading to the release of cytochrome C (*134*).

Various investigations have shown that a number of phytochemicals with antioxidant properties also possess cytotoxic effect (*135*, *136*), especially in carcinoma cells, which is effected via apoptotic pathways involving Bcl-2 family of proteins (*137*). However, the regulation of mRNA of proteins involved in apoptosis and due to toxins, and their reversal by natural antioxidants such as bio-pigments (anthocyanins and carotenoids) have not been intensely researched. Low doses of oxidants like hydrogen peroxide were also capable of inducing apoptosis. In the authors' lab, the study of the expression of Bcl-2 and Bax in Hep3B cells treated with TBH were found modulated by pretreatment with bio-pigments having excellent antioxidant properties, which may also be due to the protection offered to their mRNAs. While apoptotic cell death mediated by antioxidants is desirable in cancer chemotherapy, necrosis is considered to lead to pathologic states. Similarly antioxidant can interfere at different points of signaling and abrogate the oxidative stress induced cell death. Therefore, cumulative information on the morphological changes, activation of caspases and

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expression of apoptotic proteins gives a broad picture of the state of the cells under oxidative stress status and its amelioration by antioxidants.

Extrinsic Antioxidants

There are situations where the innate antioxidant defense of the body may not suffice for the defense against excess free radicals generated under stress conditions. Recent times have seen a radical shift of interest towards naturally derived antioxidants as sources of biologically active compounds due to their health promoting and disease preventing properties. Synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT),which had occupied the major antioxidant market for the past few decades, are now less preferred due to their possible toxicity as well as loss of consumer interest (*138*). Recent research trend is to explore radical scavengers from natural sources to develop new drugs having safer and better efficacies for prevention of diseases and aging processes. Accordingly, enormous data are continuously generated suggesting that plant foods offer significant antioxidative benefits resulting in the postponement of aging and reducing the incidences of progression of pathologies.

Dietary sources of with antioxidants are important shielding against oxidative insult. Fruits and vegetables and plant extracts are rich sources of antioxidants, they clean up the free radicals as they circulate in blood and can also enter the tissues, thus curtailing the damaging effects of free radicals. A number of antioxidants form a part of the diet viz., vitamin C, vitamin E, plant derived compounds such as polyphenols, carotenoids, flavonoids etc. Natural coloring compounds have been gaining significance in biology and medicine due to their potential health benefits. Accordingly, the pigments and other antioxidants present in fruits and vegetables have chiefly been considered responsible for inhibiting the free radical mediated reactions, thus protecting the body against the damaging effects of the free radicals and other reactive species-induced diseases such as cancer, coronary heart disease, neuro-degeneration, macular degeneration, diabetes etc. (*139*). A number of antioxidants form a part of the diet viz., vitamin C, vitamin E, plant derived compounds such as polyphenols, carotenoids, flavonoids etc.

Biopigments

With increasing reports on toxicities of synthetic colors, interest has shifted towards the use of natural colors. In India, Rule 26 of The Prevention of Food Adulteration Act, 1954 (PFA) and The Prevention of Food Adulteration Rules, 1955 & 1999 permit only those colors whether isolated from natural sources or produced synthetically in food items (Table 1).

Although in some countries the use of anthocyanin as colorant is not approved, in the U.S.A., 4 of the 26 approved colorants are anthocyanin-based viz., grape skin extract, grape color extract, fruit juice and vegetable juice. In the European Union all anthocyanin-derived colorants are recognized as natural colorants under classification E163 (*140*).

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Table 1. Natural colors permitted to be used in food in India and U.S.A.

Compound	***Country***	***Color***
Beta-carotene	India/USA	Yellow/Orange
Carotene	USA	Orange
β-apo-8’ carotenal	India	Yellow/Orange
Methylester of β-apo-8, carotenoic acid	India	Yellow/Orange
Ethylester of β -apo-8’ carotenoic acid	India	Yellow/Orange
Canthaxanthin	India	Yellow/Orange
Chlorophyll	India/USA	Green
Chlorophyllin	USA	Green
Riboflavin (Lactoflavin)	India	Yellow
Caramel	India	Brown
Annatto	India/USA	Yellow/Orange
Saffron	India	Orange
Curcumin (turmeric)	India/USA	Yellow
Capsanthin	USA	Red/Orange
Beetroot	USA	Pink/blue/red
Carmine	USA	Red
Lycopene	USA	Reddish orange
Carminic Acid	USA	Orange/Red
Lutein	USA	Yellow
Anthocyanin	USA	Red/Purple
Vegetable carbon	USA	Black

Carotenoids

They form one of the largest classes of natural pigments and are responsible for red-yellow-orange colors of most fruits and vegetables. These pigments are of interest for pharmaceuticals, coloring food/feed andas nutrient supplements due to their high antioxidant activity. Carotenoids are defined by their chemical structures. They are composed of 40-carbon polyene chain and depending on the presence of terminal cyclic end-group (phenolic) and oxygen containing functional group are classified respectively as:

- carotene hydrocarbons and
- xanthophylls (oxygenated derivatives of carotene hydrocarbons).

Naturally occurring carotenes mostly occur in all-trans form. Structures of some of the physiologically important carotenoids are presented in Figure.1. The structure of a carotenoid ultimately determines the potential biological function that the pigment may have. The characteristic pattern of alternating single and double bonds in the polyene backbone of carotenoids allows them to absorb excess energy from other molecules, while the nature of the specific end groups on carotenoids may influence their polarity. The former may account for the antioxidant properties of biological carotenoids, while the latter may explain the differences in the ways that individual carotenoids interact with biological membranes (*141*). The extensive conjugated double bond system serves as the light-absorbing chromophore thus imparting red, yellow or orange color to the compound. The extensive conjugated double bonds system is attributed to the singlet oxygen quenching capacity of carotenoids, such activity is known to increase with the increase in the number of double bonds.

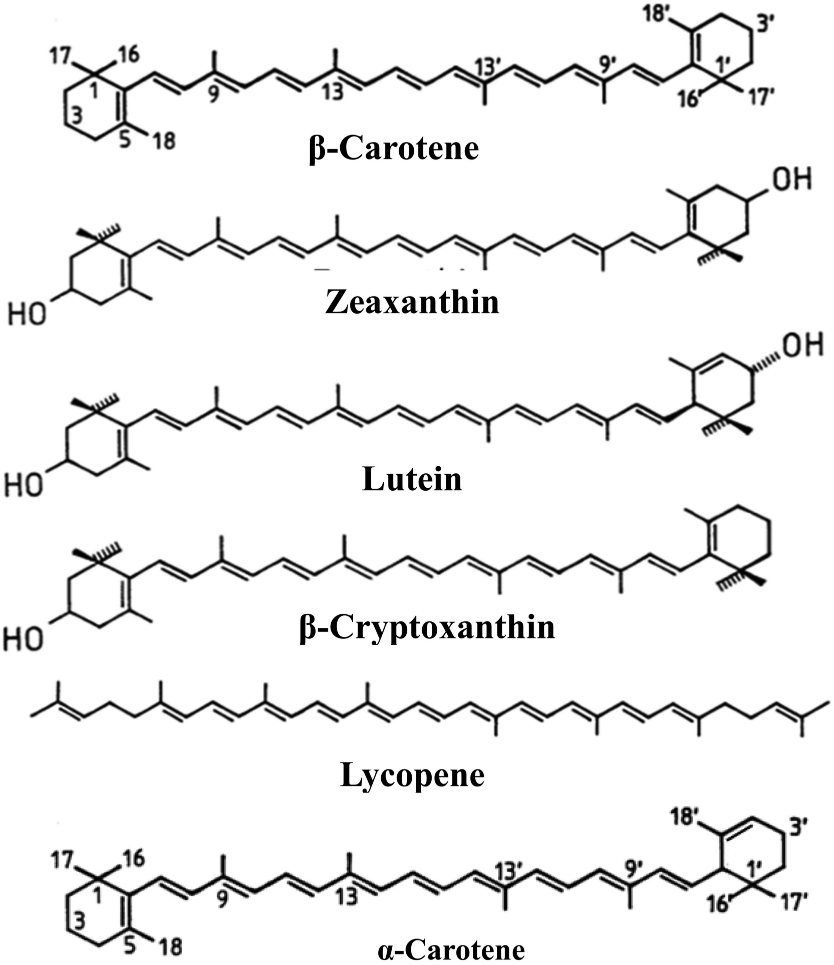

Figure 1. Chemical structures of some physiologically important carotenoids.

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While xanthophylls are predominant in green leafy vegetables, almost all carotenes are found in yellow vegetables. Carotenoids such as β-carotene, α-carotene and cryptoxanthin act as provitamin A compounds, being converted into vitamin A in vivo. β-carotene and lutein are the most common carotene and xanthophyll known. Modes of actions through which carotenoids help in the prevention of chronic diseases are depicted in Figure. 2.

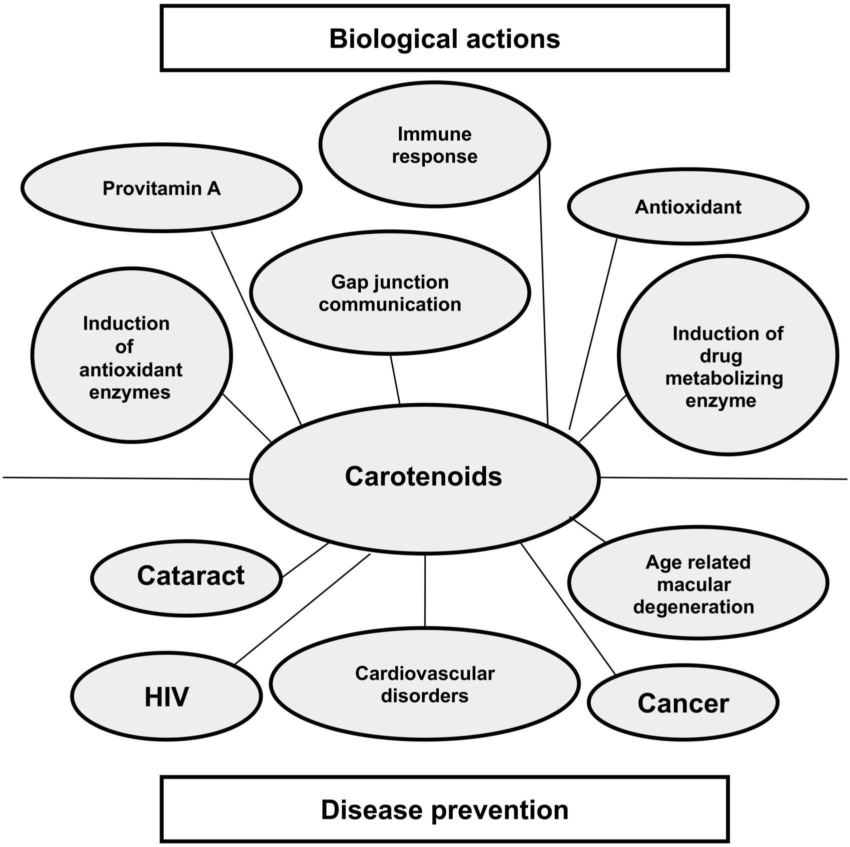
Biological actions
Immune response
Provitamin A
Antioxidant
Gap junction communication
Induction of antioxidant enzymes
Induction of drug metabolizing enzyme
Carotenoids
Cataract
Age related macular degeneration
HIV
Cardiovascular disorders
Cancer
Disease prevention

Figure 2. Role of carotenoids in the prevention of certain chronic diseases.

Carotenoids nullify the reactive nature of pro-oxidants by maintaining electron balance, and such functions are more efficiently carried out by carotene hydrocarbons than the xanthophylls (*142*). Carotenoids have been reported impart cancer chemopreventive activities in in vitro cell cultures and animal models. Lutein and zeaxanthin have been found to be independently associated with the reduced risk of age-related macular degeneration (*143*). Carotenoids have received much interest since the report of Peto et al (*144*) who suggested

In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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the role of β-carotene from fruits and vegetables as a cancer-protective agents. Although chiefly known for their provitamin-A activity and antioxidative properties, carotenoids are responsible for a wide range of intercellular activities such as communication, immune response, neoplastic transformation, growth control and regulation of cellular levels of the enzymes that detoxify carcinogens (*145*). Epidemiological studies indicate an inverse relationship between consumption of fruits and vegetables rich in carotenoids and risk of cancer and cardiovascular diseases, attributed to their antioxidant functions through singlet oxygen quenching and deactivation of free radicals (*142*, *146*). Low plasma carotenoids concentration is often used as an indicator for those at risk of chronic diseases (*147*). Such indices are based on the direct association between the intake of carotenoid-rich fruit and vegetables, plasma and tissue concentration of carotenoids and the incidences of development of chronic disease states, particularly cardiovascular diseases and cancer of various organs (*148*). Similarly, plasma xanthophylls are inversely related to the indices of oxidative DNA damage and lipid peroxidation (*149*). Natural antioxidants are more favorably accepted and recognized by higher animals than synthetic ones (*150*). The same is true with carotenoids, the natural forms of which primarily exist in the all-*trans* form (*151*) along with oxygenated carotenoids that have better efficacy over synthetic all-*trans* forms (*152*). However, there have been contradictory reports about the effect of carotenoids, specifically, β-carotene. Though protective against oxidative stress, β-caroteneis said to increase the risk of lung cancer in smokers and workers occupationally exposed to asbestos (*153*, *154*). Therefore, it is essential to carry out a detailed study to assess the effectiveness and safety of carotenoids, to define the population reaping benefits from carotenoids, to specify the dose and length of treatment and also to establish whether mixtures of carotenoids, rather than a single carotenoid, are more effective.

Carotenoids have been shown to be cytoprotective in various oxidative stress models. Carotenoids of *Dunaliella salina* show strong protection against CCl_4-induced hepatotoxicity in experimental models by increasing the activities of CAT, SOD and GPx in addition to decreasing the activities of hepatic marker enzymes (*155*). Carotenoids such as α-carotene, β-carotene, lutein and lycopene showed protection against CCl_4-induced toxicity to hepatocytes in in vitro cultures by reducing the lipid peroxidation while they also increased the cell survival (*156*). Lycopene supplementation in congestive heart disease patients showed increased serum AOE activities as compared to those patients who were not supplemented (*157*). Consumption of high amounts of fruits and vegetables, known to be rich in antioxidants, is reported to increase erythrocyte GPx activity, which is correlated to the non-nutritive antioxidants such as carotenoids, anthocyanins and flavonoids (*158*).

Anthocyanins

Anthocyanins form the largest water soluble pigments, synthesized mostly in higher plants. These are chemically grouped under flavonoids family forming a group of phenolic compounds responsible for the pink-red-purple-blue colors of foliages, flowers, fruits and vegetables, some roots and tubers. Next to chlorophyll, they are the most important group of plant pigments visible to the human eye. Chemically anthocyanins are glycosylated polyhydroxy or polymethoxy derivatives of flavilium (2-phenylbenzopyrilium) salts. The basic structure of the flavilium salt and the structures of most commonly occurring anthocyanins are depicted in Figure. 3. Glycosidic substitutions occur at 3 or 5th position which may be acylated with aliphatic acids or cinnamic acid. This leads to a considerable structural variation giving rise to about 600 anthocyains which have been reported in nature (*159*).

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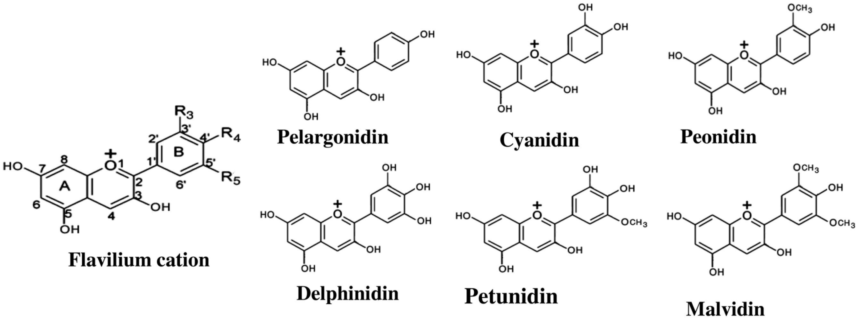

Figure 3. Chemical structure of Flavilium cation and of most commonly occurring anthocyandins.

The chemical structure of the anthocyanin plays an important role in deciding the color and also the biological function. Anthocyanins from different sources vary from each other with respect to their color and stability. This is attributed to the difference in glycosidic substitution and the presence and type of acylating molecule, their activities regulated by the position, number and type of substitution on the flavilium cation (Figure 3). Stintzing et al (*160*) reported that glycosidic substitution at 5th position of the cyanidin glycoside and acylation with cinnamic acids shifts the color to more purplish hue. Commercial anthocyanin colorants are mostly derived from fruits and vegetables the largest natural commercial source being the red grape skin. Other sources include elderberry, blackberry, raspberry, red cabbage, black carrot, purple corn, red radish and purple sweet potato.

A substantial amount of literature points to the fact that anthocyanins have been implicated in the prevention and alleviation of a number of pathophysiological conditions, owing to their strong antioxidant properties. The "French paradox" - an inverse correlation between wine consumption in French population and cardiovascular diseases- is largely attributed to the presence of anthocyanins in the red wine and a derivative phenolic, the resveratrol. Anthocyanins are a part of normal diet which comes with the consumption of fruits and vegetables and are known to possess excellent antioxidant properties (*161*). Anthocyanins from different sources have been reported to inhibit lipid peroxidation, platelet aggregation (*162*), possess antitumor, antimutagenic (*163*),

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hepato-protective (*164*) and cardio-protective (*165*) properties. Anthocyanin extracts from chokeberry and bilberry at low doses have been found to inhibit ROS and at higher concentrations induced endothelium-dependent vaso-relaxation in porcine arteries suggesting a beneficial effect in vascular disorders (*166*). Administration of black currant and bilberry anthocyanins improved visual acquity and enhanced night vision in animals and humans, while black currant anthocyanins have also been shown to stimulate rhodopsin regeneration (*167*) in the retina. Anthocyanins and ellagitannins of blackberry reversed thiobarbituric acid-induced oxidation and enriched glutathione pool in liver, kidney and brain (*167*).

In vitro and in vivo trials have demonstrated the potential of anthocyanins to prevent cancer cell proliferation and inhibit tumor formation. Hou (*168*) and Hou et al (*169*) revealed that anthocyanins inhibit tumorogenesis by blocking activation of a mitogen-activated protein kinase pathway. This study provided the first indication of a molecular basis for why anthocyanins display anticarcinogenic properties, which is mediated by their strong antioxidative mechanisms. While in other studies, fruit extracts with significant anthocyanin concentrations proved to be effective against various stages of carcinogenesis, but the individual role of anthocyanins versus other components were seldom determined. Anthocyanins are capable of forming co-pigments with DNA thus offering mutual protection against hydroxyl radicals generated by Fenton reaction (*170*). This can be a positive attribute in increasing cell survival under oxidative stress and may have physiological implications in preserving cellular functions and thus preventing its damage.

Anthocyanins of different sources have been extensively studied for their protective action against CCl_4-induced toxicity in different tissues of rodents (*164*, *171*, *172*). However, most of these reports emphasize the importance of hepatic marker enzymes rather than the AOEs and there are very few reports concerning the effect of these anthocyanins on the AOEs in stress-induced cells. It has been observed that exposure to agents that lead to oxidative stress also lead to an increase in the mRNA levels of certain AOEs. Several studies have proved that the levels of mRNAs coding for catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), as well as their activities and their mRNA stabilities are altered under conditions of diseases, and such alterations are reversed by natural extracts and plant pigments (*173*). The many anticancer properties of phytopigments listed in Table 2 could also be due to their expected but un-determined protection offered to cellular defense systems, including apoptosis, through their prevention or reversal of oxidative damage to both DNA and RNA.

In summary, sporadic studies on the integrity of cellular RNAs, particularly the stability mRNAs of antioxidant proteins such as SOD, CAT, GPX and GSH have casted light, although dim, indicating the need to focus on finding ways for the protection of the first line of oxidative defense mechanisms. Although more research is needed to study the fate of oxidized RNAs, the available data clearly indicate their cellular-damaging effects. Currently available data on plant-derived compounds, pigments in particular, clearly indicate their efficacies in reversing the pathological conditions to a significant extent, and such effects are imparted typically through reducing the burden on cellular defense system and reversal of oxidative damage by supporting cellular repair mechanisms. Enormous body of information available on the beneficial effects of natural substances and more focused research on the elcucidation of specific mechanism is expected to result in the identification of important biomolecules that are most efficient in the reversal of cellular pathological conditions and life-span extension.

Structural Differences and Functionalities of DNA and RNA

While both DNA and RNA are nucleic acids (NA), essentially similar in having building blocks of purines and pyramidines, the main difference between the two lies in the phosphorylated ribose sugar back-bone. The DNA has deoxyribose, whereas, the RNA has ribose sugar. Ribose sugar is more reactive because of C-OH (hydroxyl) bonds. Regarding individual purines and pyrimidines, the only difference is that RNA has uracil base, which pairs with adenine and uracil lacks a 5-methyl group. This is known to contribute a slightly lesser total energy of base-pairing in RNA than the higher energy of Adenine-Thymine base pair of DNA.

DNA has continuous base-pairing through hydrogen-bonding interactions, while RNA folds and forms double strands only when complementary bases can pair by hydrogen bonding, which essentially makes RNA single stranded. RNA is less stable in alkaline conditions. Larger grooves of RNA make it easier to be attacked by enzymes.

While executing cell functions, DNA transcribes complementarily to synthesize the messenger RNA (mRNA). Before leaving the nucleus, the pre-mRNA is modified by **capping,** immediately after the initiation of its synthesis, by the addition of 7-methylguanylate to the 5’end of the transcript via a 5’-to-5’linkage to protect the growing RNA transcript from degradation by RNases. When synthesis is being complete, the 3’end is cleaved at a specific site, and a **poly-A tail**, usually consisting of about 100 to 200 adenylic acid residues, is added by the enzyme poly-A polymerase. Thus, to exit the nucleus, an mRNA must possess a 5’cap and a poly-A tail, and it must be properly spliced. Incompletely processed transcripts remain in the nucleus and are degraded. The poly-A tail has several functions: (1) it protects against RNases and therefore increases the stability of mRNA molecules in the cytoplasm, (2) both poly-A-tail and the 5’guanylate cap are required for transit through the nuclear pore, and (3) it assists in its own transportation towards the ribosome for translation into peptides. In addition to RNA turnover, the **translatability** of mRNA molecules is variable. The mRNAs fold into molecules with varying secondary and tertiary structures that can influence their accessibility for efficient translation.

Another factor that can influence translatability of an mRNA is codon usage. If a message contains a large number of triplet codons that are rare for that cell, the small number of charged tRNAs available for the transportation of those codons will slow down translation, thereby increasing the chances of exposure of mRNA to oxidative stress. Added to this, the mRNAs are distributed throughout the cytoplasm with their many sections being single stranded, making them more

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amenable for oxidative damage resulting in the turn-over of wrong proteins. Finally, the **cellular location** at which translation occurs seems to affect the rate of gene expression. Free polysomes may translate mRNAs at very different rates from those at which polysomes bound to the endoplasmic reticulum do; even within the endoplasmic reticulum, there may be differential translation rates. RNA may simply interfere with a cell's normal activities and/or it may induce checkpoints leading to apoptosis

Table 2. Summary of anticancer properties of flavonoids and carotenoids through their antioxidant effects on nucleic acids, lipids and proteins

Class	*Compound*	*Anticancer activity*
Flavonoids	Anthocyanins	Delphinidin, cyanidin, and petunidin have been shown to inhibit chemical carcinogen-induced AP-1 transcriptional activity and cell transformation in JB6 cells (*169*, *174*). Anthocyanins inhibit tumerogenesis by blocking the activation of mitogen-activated protein kinase (MAPK) pathway and c-jun NH_2 terminal kinase (JNK) which is ROS dependent (*169*, *175*). Anthocyanins from black raspberries prevent oesophageal tumors in rats probably by down-regulating carcinogen-induced nuclear factor-κB (NF-κB) and activator protein-1 expression (*176*). While being non-toxic to normal cells, anthocyanins induce peroxide accumulation and apoptosis in HL-60 cells (*175*).
	EGCG	In tumor cells, EGCG induces production of H_2O_2, creating an oxidative stress environment (*177*, *178*) which triggers an apoptotic pathway which is distinct from chemical or Fas-mediated pathways and acts through activation of mitogen-activated protein kinases, c-Jun N-terminal kinase and p38, and the caspase cascade (*178–181*).
	Quercetin	At low doses (0.1-5μM), it shows cytoprotection while at higher doses (50-100 μM) it is clearly cytotoxic (*106*). It inhibits cell proliferation by down-regulating the cell cycle genes such as CDC6, CDK4 and cyclin D, inducing cell cycle arrest and by up-regulating tumor suppressor genes (*182*). It stimulated proliferation of colon carcinoma cells at lower concentrations while at higher concentrations inhibits the same (*183*, *184*).

Continued on next page.

Table 2. (Continued). Summary of anticancer properties of flavonoids and carotenoids through their antioxidant effects on nucleic acids, lipids and proteins

Class	*Compound*	*Anticancer activity*
Carotenoids	β-carotene	β-carotene induces ROS production and activation of NF-κB, by a redox mechanism which is accompanied by inhibition in cell growth and induction of apoptosis through over expression of c-myc, an apoptosis inducing protein, in leukemia and colon cancer cell lines (*185*). It has also been found to exhibit anticancer activity in breast cancer cells by down-regulation of cycloxygenase-2 (COX2) mediated by the up-regulation of peroxisome-proliferator activated receptor-γ (PPAR-γ) and modulating the expression of its downstream components ultimately leading to apoptosis through ROS production (*186*). However, β-carotene consumption in high doses is presumed to increase risk of lung cancer in smokers and asbestos workers (*153*, *154*).
	Lycopene	A significant inverse correlation has been found between consumption of tomatoes, a rich source of lycopene, and cancers such as that of prostate, breast, cervical, ovarian, liver and other organs (*187*, *188*). In prostate cancer, it acts by substantially reducing the levels of prostate specific antigen and also oxidative DNA damage (*189*).
Xanthophylls	Lutein	Selectively induces apoptosis in transformed mammary cells by increasing the BCl-2/Bax ratio (*190*), expression of p53 and reducing angiogenesis in the tumors (*191*) and reduces oxidative stress on retinal neurons (*192*).
	Zeaxanthin	While not many studies demonstrate its role in anticancer studies, it has been reported that zeaxanthin and lutein protect the eye retina from the damaging effects of light, thereby reducing the risk of age-related macular degeneration and cataract (*193*).
	β-cryptoxanthin	High levels of β-cryptoxanthin are associated with a reduced risk of lung cancer according to a Chinese cohort study (*194*).

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Chapter 29

Thylakoids Promote Satiety in Healthy Humans. Metabolic Effects and Mechanisms

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Thylakoids are the photosynthetic membranes of the chloroplasts in green leaves. Thylakoids have been found to promote satiety when added to food, both in animal experimental models and in human. The thylakoids act through inhibition of lipase-colipase catalysed hydrolysis of triacylglycerol, which is the main dietary fat component. The mechanism for inhibition is either a binding of thylakoids to lipase-colipase, which thereby prevents to act as a lipolytic enzyme complex or binding of thylakoids to the triacylglycerol droplet, thereby hindering the access of lipase-colipase to its substrate. Thylakoids consist of proteins and lipids in a membrane structure containing various protein-bound pigments. The thylakoid membranes are fairly resistant to gastrointestinal breakdown, which may be an important property to explain the satiety promoting effect. Satiety is promoted through the release of cholecystokinin, a gastrointestinal hormone that causes an inhibition of gastric emptying and stimulation of satiety mechanism in the brain. The hunger hormone ghrelin is suppressed as well as insulin. In human short-term experiments thylakoids added to food promote satiety signalling. In long-term a reduced body fat mass was observed.

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Introduction

The greatest Cultural Revolution in modern time occurred around 1970. At that time fast food was introduced being highly palatable and available at low cost on many places. At the same time obesity started to increase. The obesity started in USA, then spread to Western Europe and is now increasing in Asia. The incidence of obesity correlates with introduction of modern Western fast food, being energy-dense and containing fat and sucrose (*1*). Globally one billion of persons are overweight (BMI > 26) and 300 millions obese (BMI >30). According to the National Health and Nutrition Examination Survey around 40 % of the population in US is obese (*2*, *3*). In Sweden around 2,5 million people are overweight and 400 000 obese. In Sweden around 40 000 obese patients are treated with medical preparations to reduce their body weight, thus only a small percentage in relation to the number of obese subjects.

Obesity induces various diseases like diabetes, hypertension, cardiovascular disease, cancer and depression. There are great social costs for loss of work among the obese subjects as well as for the treatment, which is often life-long. There are therefore strong arguments to combat obesity (*4*).

Cause of Obesity

Obesity is an imbalance in energy intake in proportion to energy expenditure. Energy expenditure has not changed during the same period, indicating that overeating is the main cause for obesity. Under normal conditions food intake is normally balanced with energy expenditure (*5*). This occurs through the action of appetite regulating signals, acting to regulate food intake in proportion to energy needs. Appetite control occurs through the action of hunger and satiety signals (*6*, *7*). Obesity has been linked to either an over expression of hunger signals, a low expression of satiety signals, or an inadequate regulation of these.

A reduced satiety signalling leads to overeating and to suppressed cellular nutrient uptake and oxidation. Such a reduced satiety signalling is found in obese subjects, particularly in relation to palatable food (*8*, *9*). Palatable food is highly concentrated on energy content. Volume of food is an important factor in regulating satiety. With highly energy-dense food, the satiety component is further reduced (*7*). Adding volume through liquids does not produce satiety; the volume must instead be included in the food eaten (*10*). The highly palatable food eaten today hence does not produce satiety and therefore overeating leads to an expanded fat mass and obesity.

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Hunger Signals

During energy deficiency hunger signals are released that trigger food intake (*11*). A low blood sugar level is the most obvious sign of energy deficiency of the body. A low blood sugar level releases several hunger signals, acting to restore energy balance. Hunger involves three processes: 1) food seeking 2) food intake and 3) food storage. One important hunger signal is ghrelin, released from the stomach when the stomach is empty (*12*). Ghrelin levels are raised during fasting and suppressed within 30 minutes after the start of a meal. Obese subjects often have a blunted response to food; the ghrelin levels are not adequately suppressed. Obese children with Prader-Willis disease have significantly elevated levels of ghrelin levels, which may explain their overeating. Carbohydrate is more efficient to suppress ghrelin levels after feeding, whereas fat is least effective and protein in between (*13*). This could explain why fat easily promotes overeating.

Satiety Signals

The satiety signals act to promote satiety. Their function could be summarised in three processes: 1) termination of eating 2) stimulation of uptake of nutrients into the cell and 3) stimulation of utilization of body energy stores during fasting. Following ingestion of food satiety signals are released. These are released in the intestine and serve to induce an early satiety during a meal. Satiety signals from adipose tissue serve the function to act satiety between two meals, thus regulating inter-meal satiety.

Termination of eating occurs through the action of gastrointestinal satiety hormones, such as cholecystokinin (*14*), glucagon-like peptide1 (*15*) and enterostatin (*16*). These are released when food enters the intestine and produce satiety through activation of reward molecules such as serotonin, which brings the body into a state of rest. A stimulated uptake of nutrients is particularly important for liver cells, where fuel sensing occurs and where inter-meal satiety is regulated (*17*). A stimulation of energy expenditure during fasting is important to keep the energy levels of the cell at a level sufficient for basal function (*18*). This occurs through activation of glucose and fat oxidation. A strengthening of satiety will act both to promote a decreased food intake and to stimulate energy expenditure.

Satiety with Green Leave Components (Thylakoids)

We have in our experiments identified certain components from the green leave spinach, so called thylakoids that produce satiety. The satiating response has been documented in experimental animal models as well as in human studies.

Thylakoids are those cell structures within the plant cell that is responsible for the light reaction of photosynthesis (*19*) (Figure 1). Thylakoids consist of proteins and lipids, 50 % of each. In addition they contain chlorophyll and various antioxidants like carotenoids and vitamin E (*20*). The lipids are mainly polyunsaturated of omega-3-type and most of these in the form of galactolipids. Thylakoids are the most common type of biological membrane and therefore have a great potential as source for functional food components. The thylakoids have

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no taste when harvested and can be rapidly isolated. A large-scale procedure for the preparation of purified thylakoids have been described where the thylakoids are precipitated at pH 4.7 , the iso-electric pH of thylakoids, to increase the yield of material (*21*).

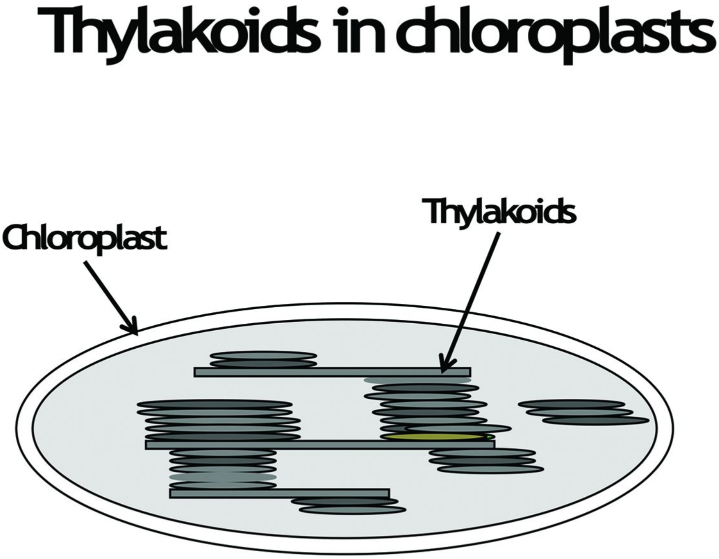

Figure 1. Thylakoids are membranes responsible for the light reaction of photosynthesis in the chloroplasts of green plants. They contain membrane lipids, hydrophobic intrinsic membrane proteins and pigments necessary for the photosynthetic process.

Animal Studies

Starting with animal studies in rat we demonstrated that thylakoids when added to high-fat food at a concentration of 2 mg chlorophyll per gram food significantly suppressed food intake and caused a reduced body weight by 18,5 % (*22*). The reduction of food intake started a few days after the thylakoids were given into the food, suggesting that the thylakoids did not have any aversive effect, which otherwise would have acted immediately. Instead there was a gradual inhibition of food intake with thylakoids, revealing that the satiety signals were gradually upregulated. The promotion of satiety was explained by raised levels of the satiety hormone *cholecystokinin*, being significantly elevated from 0,086 ± 0,12 pmol /l to 0,675 ± 0,08 pmol /l in the thylakoid group compared to the control group. Cholecystokinin (CCK) is a gastrointestinal hormone that ensures proper digestion and absorption of food through stimulation of pancreatic juice and bile and through inhibition of gastric emptying. CCK is released by fatty acids and amino acids as long as food digestion is going on and ceases after the aborption of the food products by the intestine (*23*). CCK in addition to peripheral effects in the intestine also has central effects in the brain, with a

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stimulated release of serotonin, a reward molecule that is important for central satiety (*24*). A stimulated release of CCK suggests that the food processing in the intestine is slowed down by thylakoids and that brain satiety is induced.

In addition to a reduced body weight we observed reduced levels of circulating triacylglycerol levels from 1,02 ± 0,13 mmol /l in control animals to 0,62 ± 0,04 mmol /l in the animals receiving thylakoids in the food. An elevated level of triacylglycerol is a common phenomenon in obese subjects with a great fat mass. The elevation suggest that the blood lipids are either not taken up by the peripheral cells or that they are not utilized. We cannot at present time explain the reduced triacylglycerol levels, but this is a phenomenon that we have observed in other experiments both in animals and in humans following addition of thylakoids in the food. The satiety signals released by thylakoids probably stimulate the uptake and utilization of triacylglycerol. The reduction of triacylglycerol levels is important to prevent any aberrant accumulation of lipids in other tissues, including the arterial wall where it causes atherosclerosis, in pancreas, where it causes diabetes or in liver, where it causes deregulation of the liver.

In further animal experimental studies we gave mice a thylakoid-enriched high-fat diet during 100 days, and control mice were given a high-fat diet (*25*). During this feeding paradigm the mice receiving thylakoids had a significantly reduced food intake and reduced weight gain by 17 % compared to control mice. The effect started not until 30 days of feeding, suggesting that the weight reducing effect was gradually set up in the animals. The animals also had a significantly reduced body fat mass by 33 % and reduced triacylglycerol levels by 25 %. The reduction of triacylglycerol levels correlates with a reduced body fat mass in the animals receiving thylakoids. The thylakoid-treated animals also had reduced serum glucose levels by 19 % and reduced fatty acid levels by 17 % compared to control animals, suggesting a better metabolic control. High-fat feeding often leads to hyperglycaemia, hyperlipidemia and an increased fat mass, effects that thus were prevented with thylakoids.

Also in the mice treated with thylakoids the fasting levels of the satiety hormone CCK was elevated at the end of the experiment by 65 % (*25*). CCK is a satiety hormone released as long as intestinal digestion is going on; the elevated levels suggest that the animals had a prolonged time for food digestion. The release of CCK seemed specific, since another gastrointestinal hormone, PYY, was not released. PYY is produced in the lower part of the gut, whereas CCK is produced in the upper part (*26*). This suggests that the delayed fat digestion occurs in the upper small intestine and is not as severe as should be expected when digestive enzymes are lacking, where digestion could continue all along the intestine, even at distal ileum (*27*). Thus thylakoids act to slow down fat processing temporarily, the main effect being in the upper small intestine.

Thylakoid Effect in Man

We also examined if human was affected by thylakoids added to food (*28*). After an overnight fasting healthy humans were served a breakfast containing various doses of thylakoids (between 5 and 50 grams of thylakoid powder) added

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as a pesto sauce on a sandwich, eaten together with tomatoes and basil. Blood samples were taken during six hours and then analyzed for various hormones. It was found that thylakoids promoted the release of CCK in a dose dependent way, the optimal dose being 25 gram of thylakoid, which gave an increased CCK level from 0, 5 ± 0,15 pmol /l to 1,3 ± 0,20 pmol /l at time point 6 hours. Whereas control breakfast gave a CCK release that raised within one hour after start of feeding and was at fasting level after four hours, the thylakoid enriched breakfast gave a release of CCK after one hour, and stayed elevated for six hours, being significantly elevated compared to the control. This suggests that thylakoids promote satiety also in man. The prolonged satiety was also obvious from the appearance of free fatty acids in the circulation. During the control meal free fatty acids appeared in the circulation after three hours, whereas in the thylakoid meal the free fatty acids had not appeared in the circulation after six hours. Free fatty acids emerge in the circulation when energy is needed and in a state of hunger. With thylakoids the satiety was still acting after three hours and remained at this state after six hours.

The hunger hormone ghrelin was significantly suppressed by thylakoids by 25 % compared to control feeding in man (*28*). Ghrelin is elevated during fasting and suppressed by feeding. A suppression of ghrelin levels means less hunger and could be an effect of raised CCK levels, CCK being known to reduce ghrelin levels (*29*). Normally ghrelin is most efficiently suppressed by carbohydrate in the food and least by fat. It may be that not only fat is more slowly processed by thylakoids but also carbohydrate, the carbohydrates remaining in the intestine acting to suppress the ghrelin response. Suppressed ghrelin levels mean less hunger and less craving for reward, ghrelin being an important hormone for reward seeking (*30*).

The satiety hormone leptin was significantly elevated by thylakoids, when measured six hours after feeding compared to control feeding in man, by around 40 % (*28*). Leptin is normally involved in prolonged fasting such as over-night fasting where it promotes satiety and fat oxidation (*31*). An elevation of serum leptin levels following a meal has been observed after six-hours, suggesting leptin to act also during inter-meal satiety, providing this reaches a six-hour interval (*12*). Raised leptin levels by thylakoids suggest that either satiety is promoted or that fat oxidation is promoted prior to next meal (*32*).

To our surprise also insulin levels were reduced by thylakoids compared to control in the human feeding experiment by around 37 % (*28*). This suggests that thylakoids also affect glucose uptake and glucose metabolism. Glucose levels in the blood were not different during the thylakoid meal compared to the control meal. The observation of reduced insulin levels hence suggests increased insulin sensitivity. This could be due to the suppressed secretion of ghrelin, since ghrelin is known to reduce insulin sensitivity (*33*). It appears that thylakoids influence both lipid- and carbohydrate metabolism.

The Mechanism of Action of Thylakoids

The mechanism of the thylakoid action is that they reduce the rate of fat digestion as demonstrated in vitro (*22*). The enzyme mainly responsible for

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intestinal fat digestion is pancreatic lipase and its cofactor, pancreatic co-lipase. Pancreatic lipase is inactive and unable to bind to its triglyceride substrate under the conditions of the gastrointestinal tract, i.e. in the presence of bile salt and phospholipids. However in the presence of colipase lipase is active (*34*). This occurs through the binding of colipase to the triglyceride interface and a simultaneous binding of lipase (*34*). Colipase hence anchors lipase to its triglyceride substrate, which is subsequently hydrolysed. The tertiary structure of the lipase-colipase complex has been determined and it is clear that the complex exposes a hydrophobic surface that fits to the hydrophobic triglyceride substrate (*35*). Both colipase and lipase contribute to the hydrophobic surface, which is necessary for the subsequent hydrolysis of fat. Colipase deficiency has been identified in humans demonstrating steatorrhea and also in knockout animals, which suffered from a severely impaired fat digestion (*27*, *36*). The binding of thylakoids to the lipase/colipase complex could cause the inhibition of lipolysis.

Inhibition of Lipolysis

Thylakoid membranes were found to inhibit pancreatic lipase-colipase activity in a dose-dependent way (*22*). Other biological membranes like mitochondria, plasma membranes and bacterial membranes also inhibited lipolysis. Thus this inhibiting effect of thylakoids seems to be a general effect of biological membranes. We also separated the proteins from the lipids in the thylakoid membranes (*22*). This demonstrated that the protein fraction had the most important inhibiting property. After trypsin treatment the external protein loops of thylakoid membranes were removed; the remaining fragments still had the property to inhibit lipolysis, suggesting that the membrane spanning regions of the intrinsic proteins of thylakoids possessed the inhibitory effect. One of the major membrane proteins, LHCII, light harvesting complex II, of thylakoids was isolated and found to inhibit lipolysis. LHCII contains four hydrophobic membrane-spanning loops and a synthetic peptide identical with one of these also inhibited lipolysis although not as efficient as the whole protein. Other hydrophobic proteins, like the *cytochrome bf complex* and *transhydrogenase* of thylakoids were also able to inhibit lipolysis (*22*). In contrast the water-soluble protein serum albumin had no inhibiting capacity on the lipase-colipase catalysed hydrolysis of fat, confirming previous studies (*37*).

Binding of Thylakoids

To understand the mechanism for inhibition of lipolysis the binding of thylakoids to lipase-colipase and the oil droplet was investigated. It was found that the thylakoids strongly bound to the lipase-colipase complex (*22*). The binding probably occurs at the hydrophobic surface, normally responsible for the binding to the triglyceride substrate. In this way lipolysis thus could be inhibited. However, thylakoids also bound to the triacylglycerol surface, the thylakoids having a strong affinity for the oil phase (*22*). Calculation of the surface of thylakoids in comparison with the surface of the oil droplets in our in vitro assay demonstrated that the thylakoids actually covered the surface of the oil droplet/triglycerides. With such a mechanism the thylakoids would hinder access of the lipase-colipase complex to the interface. There is probably a complex interaction between the lipase, colipase, bile salt, oil droplet and thylakoids and it is at present time not possible to determine whether the binding of thylakoids to lipase-colipase is the most important property to explain the inhibited lipolysis or the binding of thylakoids to the oil droplet.

Satiety Following Inhibition of Lipolysis

We have in previous studies demonstrated that inhibition of the lipase-colipase mediated hydrolysis of fat leads to a promoted satiety and a reduced body weight gain in experimental animal models (*38*). The inhibition lead to reduced food intake, raised CCK-levels and reduced triacylglycerol levels. In those studies we also compared with the lipase inhibitor Xenical, which is clinically used for treatment of obesity. Xenical induces steatorrhea but does not promote satiety signalling (*39*). The explanation is the rapid passage of the fatty intestinal content during production of fatty stools. With thylakoids the food digestive products remain in the intestine until they are digested; this means that satiety signalling is promoted, at least from the upper small intestine. The absorption of the food products has probably occurred in the proximal intestine, since distal hormones were not affected.

Thylakoids in the Gastro-Intestinal Tract

The thylakoids are eventually broken down by gastro-intestinal enzymes and absorbed as nutrient products (*40*). The breakdown of thylakoids is however remarkably slow. In an experiment thylakoid membranes as well as plasma membranes were treated with gastrointestinal proteases, and the breakdown products analysed by gel electrophoresis and mass spectrophotometer. It was found that thylakoids remained resistant for two hours at 37 °C, whereas the plasma membranes were degraded within 10 minutes. Also delipidated thylakoid membranes were rapidly degraded, suggesting that the pigments contained in the thylakoids membrane proteins were responsible for the resistance toward proteolysis. Addition of oil into the incubation further enhanced the protease resistance of thylakoids. There are quite a few proteins that inhibit pancreatic lipase-colipase activity in vitro. Unpublished studies have demonstrated that these proteins (casomorphin or lactoglobulin) does not promote satiety or reduced body weight gain in vivo during feeding experiments. The explanation is probably that these proteins are rapidly hydrolysed in the gastrointestinal tract. With such proteins added to food the inhibition of lipase-colipase is only transient and does not affect fat digestion, food processing and satiety.

Mitochondria strongly inhibit lipase-colipase in vitro (*22*) and when added to food failed to reduce body weight gain in growing mice. This suggests that mitochondria are not resistant to gastrointestinal hydrolysis, probably because they contain much less pigments than thylakoids. Mitochondria-rich food such as red

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meat has however been found helpful to control energy balance, reduce abdominal fat mass and waist circumference in human epidemiological studies, suggesting that there may be some reduction of fat digestion and promotion of satiety in man (*41*).

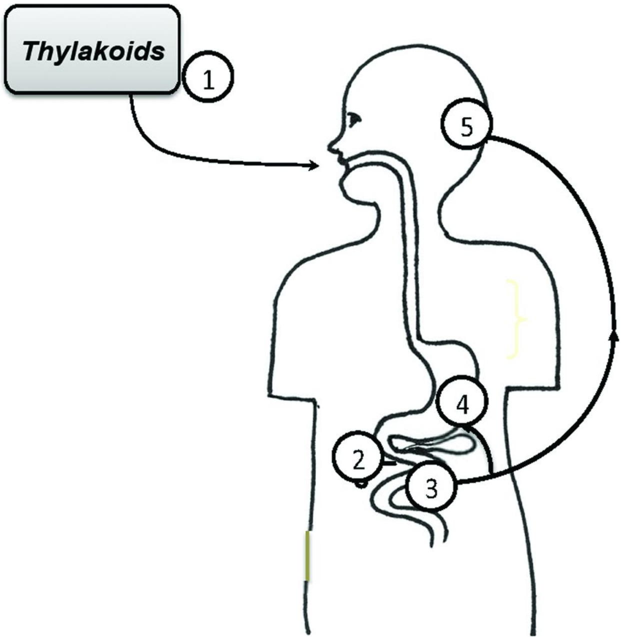

Figure 2. Mechanism of action of thylakoids in regulating appetite. Thylakoids are added to food (1), causing a delayed fat digestion in the intestine (2). The delayed process of digestion causes an increased release of the satiety hormone cholecystokinin (CCK) (3). CCK causes a peripheral inhibition of gastric emptying, hence casing a distension of the stomach (4). CCK also causes the release of rewarding satiety molecules like serotonin, in this way establishing a central reward/satiety (5).

Future Experiments

Obesity results from a prolonged small positive energy imbalance, and treatment needs to reverse this imbalance. Many different diets have been tried to treat obesity, and weight loss occurs with all of them. There is currently no evidence that supports the superiority of one macronutrient composition for diets over any other. The principal effect seems to be the degree of adherence to the prescribed calorie reduction. Obesity drugs have been developed that tap brain mechanisms for controlling feeding and the gastrointestinal tract and its peptides.

Future experiments will be performed where overweight and obese subjects will be given thylakoids to the daily food or as capsulas served with each meal. The dose of thylakoids will be tested, but should be around 5 gram of thylakoids each day.

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Conclusions

Appetite regulation with palatable food is a difficult issue, mainly since such type of food is rapidly digested and easily absorbed by the intestine. Through the addition of thylakoids from green leaves the food digestion is delayed enabling gastrointestinal satiety mechanism to work, both peripherally and centrally, as summarized in Figure 2. Future experiments will tell whether the addition of thylakoids to the daily food is an efficient treatment for overweight or obesity.

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Chapter 30

Nutrition in the Treatment of Dry Eye with Special Attention to Sea Buckthorn Oil

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Dry eye is a common condition associated with ocular inflammation and discomfort. The risk of dry eye is increased in women and in older age, and is affected by several external factors including contact lens wear and certain medications. Published studies suggest that intake of n-3 fatty acids and/or oils rich in both γ-linolenic and linoleic acids beneficially affect dry eye. Deficiency of vitamin A may be a risk factor. Benefits of antioxidant supplementation have been reported. Sea buckthorn (*Hippophaë rhamnoides*) is a traditional health-promoting plant. The combined seed and pulp oil of sea buckthorn is rich in carotenoids, tocopherols and tocotrienols and has a linoleic:linolenic acid ratio of ≤2. Intake of the combined sea buckthorn oil positively affected the symptoms of dry eye, as observed in a double-blind study of 100 women and men. It also restricted the rise in tear film osmolarity, a central indication of dry eye. The fatty acid composition of the tear film was not affected. Instead, the effects were likely to be mediated *via* attenuation of inflammation and oxidative damage.

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Dry Eye

Dry eye is a multifactorial disease of tears and the ocular surface resulting in symptoms of discomfort, visual disturbance, and tear film instability. The International Dry Eye Workshop in 2007 emphasized the role of ocular inflammation and increased tear film osmolarity as common to different forms of dry eye (*1*).

Tear film lubricates and protects the ocular surface and provides it with nutrients. It is composed of three continuous layers: a hydrophilic mucous layer anchored to surface epithelial cells and produced by conjunctival goblet cells; an intermediate protein-rich aqueous layer produced by the main and accessory lacrimal glands, and; a hydrophobic lipid layer produced from the meibomian gland extraction, meibum (*2*, *3*).

In the aqueous-deficient dry eye the lacrimal secretion is reduced. There may be several reasons for this, including obstruction or dysfunction of the lacrimal ducts, or reflex hyposecretion (*1*). Sjögren's syndrome patients commonly suffer from dry eye. Sjögren's syndrome is an autoimmune disease of the exocrine glands, characterized by lymphocytic infiltration to the lacrimal and salivary glands. Inflammation in the lacrimal glands causes acinar and ductular cell death and the hyposecretion of tears (*1*, *4*).

In the evaporative dry eye the evaporation of the aqueous tear film is excessive. This may be due to dysfunction of the meibomian glands secreting the lipid rich-meibum. The meibum lipids form the outermost layer of the tear film and restrict the evaporation of the aqueous tear (*1*, *5*). In patients with meibomian gland dysfunction, the fatty acid composition of the tear film lipids differs from that of healthy people (*6*). In addition, the lipid layer of the tear film is commonly thinner in people experiencing dry eye symptoms compared to those having no symptoms (*7*).

Both aqueous deficient, and evaporative dry eye cause hyperosmolarity of the tear film. Hyperosmolarity activates inflammation and induces damage to the surface epithelium. The loss of mucin-producing goblet cells, among others, leads to instability of the tear film. This further fortifies the hyperosmolarity and induces more detrimental changes (*1*). Typical symptoms include soreness, dryness, grittiness, burning, and redness of the eyes, as well as watery eyes or blurry vision (*8*). The risk of dry eye is increased by several external factors including contact lens wear, windy and dry conditions, and tobacco smoke. The condition is more common in women than in men and its prevalence increases with age (*5*, *9*). As androgens regulate the functions of the meibomian gland, this increase in the risk of dry eye may be due to androgen deficiency in aging people (*10*).

Given the multifactorial nature of dry eye and the poor association between the signs and symptoms of dry eye, a combination of diagnostic tests are recommended for diagnosis. Focal clinical tests include tear film osmolarity (mOsm/L), tear film stability as tear film break-up time (TBUT, seconds until break up of fluorescein tear film, observed under slit lamp), tear secretion analysis using the Schirmer test (mm of tear flow in strip of filter paper placed under the lower eyelid in 5 min), symptom questionnaires and cytological analyses (*9*, *11*).

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Vitamin A and Antioxidants in the Treatment of Dry Eye

Vitamin A affects the regulation and differentiation of epithelial cells, including those in the eyelids, conjunctiva, and cornea. Accordingly, vitamin A deficiency is detrimental, and may predispose to dry eye. Loss of goblet cells has been observed due to the lack of vitamin A. Lacrimal glands contain vitamin A in the form of retinyl palmitate, whereas the tear fluid contains retinol (*5*, *12*, *13*). Topical application of vitamin A has been shown to beneficially affect dry eye in humans. In a prospective, randomized, controlled, parallel group study the vitamin A treatment significantly improved blurred vision, TBUT, the Schirmer test score and the cytological parameters (*12*).

Carotenoids with β-ionone ring function as precursors for vitamin A, and may via this mechanism beneficially affect dry eye. Also the antioxidant and anti-inflammatory activity of carotenoids (including those that are not precursors of vitamin A) (*14*) may interfere with mechanisms of dry eye. In patients with meibomian gland dysfunction the amount of carotenoids in the meibum is reduced. The decline in meibum carotenoids is associated with older age (*15*).

The ocular surface is exposed to radiation, atmospheric oxygen and environmental chemicals, which makes it vulnerable to damage caused by the reactive oxygen species. Oxidative damage may contribute to the activation of inflammation and dry eye (*16*). Peponis et al. (*16*) reported the beneficial effect of vitamin C (1000 mg/d for 10 days) and vitamin E (400 IU/d for 10 days) on eye health, as judged by the Schimer score and TBUT in diabetic patients. Blades et al. (*17*) investigated the effects of an antioxidant supplement in a prospective, randomized, placebo-controlled cross-over study of 40 participants. Consumption of the product, containing several antioxidants (β-carotene, flavonoids), vitamins (E, C, D, B6, B1, B2, B12, K, folic acid, pantothenic acid), minerals, and amino acids for one month significantly improved the stability of the tear film and the health of the ocular surface compared to the baseline. No changes were observed in the placebo group during the intervention. The increase in the tear stability correlated with the increase in the density of goblet cells in the conjunctiva.

Dietary Oils in the Treatment of Dry Eye

Long chain n-3 fatty acids are precursors of anti-inflammatory eicosanoids. They may affect the expression of inflammatory genes (*18*). Dihomo-γ-linolenic acid (20:3n-6), a metabolite of γ-linolenic acid (18:3n-6), is a precursor of prostaglandin E_1 (PGE_1), which has anti-inflammatory and anti-aggregatory properties and may induce the production of tears (*19–21*). The effects of the eicosanoids produced from arachidonic acid (20:4n-6), the more unsaturated derivative of dihomo- γ-linolenic acid, are in general considered pro-inflammatory and pro-aggregatory (*18*, *22*).

Supplementation with a combination of linoleic (18:2n-6) and γ-linolenic acids has been reported to beneficially affect dry eye in humans (*21*, *23–26*). Barabino et al. (*26*) reported the positive effects of oral oil intake for 45 days in a randomized, placebo-controlled trial of 26 participants. The source of the oil was not specified in the article, but the product contained 57 mg of linoleic acid and

30 mg of γ-linolenic acid in each daily dose. The parameters that were positively affected included dry eye symptoms, ocular inflammation, and the lissamine green test score, which reflects the presence of non-vital cells in the cornea and conjuctiva.

The same oil preparation (*26*) at a 50% lower dosage was used in the study by Macri et al. (*24*). They investigated the effects of linoleic and γ-linolenic acids in participants undergoing photorefractive keratectomy, which is a known risk factor for dry eye. In the study of a total of 60 participants the group receiving the active oil supplement showed improvement in their ocular symptoms, a higher tear fluorescein, and an increase in tear production compared to the control group.

Pinna et al. (*23*) studied the effects of the same preparation using a daily oral dose of 28.5 mg of linoleic acid and 15 mg of γ-linolenic acid. The 57 patients with meibomian gland dysfunction in their study were divided into three groups: one treated using eyelid hygiene, one treated with the oil capsules and one treated with a combination of eyelid hygiene and oil capsules for 180 days. The symptoms of dry eye were significantly improved in all treatment groups. Oil intake significantly reduced the turbidity of the meibomian secretion and meibomian gland obstruction, suggesting that the composition of the meibum may have been affected. The effect of the oil was further improved by eyelid hygiene, as other signs were also positively affected in the combined oil and hygiene group (*23*).

Aragona et al. (*21*) investigated the effects of combined linoleic (224 mg/d) and γ-linolenic acids (30 mg/d) in patients with Sjögren's syndrome. In a double-blind, placebo-controlled study of 40 participants, the oil intervention for one month significantly increased the levels of prostaglandin E_1 (PGE_1) in tears. After the oil intake was discontinued, the PGE_1 concentrations started to decline. The oil positively affected the symptoms of dry eye and corneal fluorescein staining (*21*).

The report by Kokke et al. (*25*) is an exception among such studies in that it specified the oil that was used as a source of linoleic and γ-linolenic acids. Most other studies do report the main fatty acids, but the plant/animal source is not mentioned. The group of Kokke et al. (*25*) investigated the effects of evening primrose oil on contact lens-associated dry eye in a study of 76 women. The participants consumed ≈3 g of evening primrose oil or olive oil as a placebo daily for six months. Evening primrose oil improved the overall lens comfort and reduced the feeling of dryness. Among the objective diagnostic tests performed, the tear meniscus height was increased but the other test scores were unaffected.

In patients with Sjögren's syndrome, however, supplementation with 800 mg or 1600 mg γ-linolenic acid from evening primrose oil did not improve in the symptoms of dry eye or Schirmer test results. The intervention period in the double-blind placebo-controlled study of 90 patients lasted for 6 months (*27*).

In an epidemiological study (*28*), the high dietary intake of n-3 acids was associated with a reduced risk of dry eye in women. No independent effect of n-6 intake on dry eye was observed. A high n-6/n-3 ratio was associated with an increased risk. In this study the association of individual n-6 fatty acids and dry eye was not analyzed. In an animal study, a combination of n-3 fatty acids and

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γ-linolenic acid was superior for the treatment of dry eye compared to n-3 or n-6 fatty acids alone (*28*, *29*).

Positive trends due to the intake of n-3 fatty acids were recently observed in a randomized, double-blind, placebo-controlled study of 36 dry eye patients. The participants consumed a combination of fish oil (450 mg eicosapentaenoic acid (20:5n-3) and 300 mg docosahexaenoic acid (22:6n-3)) and flaxseed oil (1 g) or wheat germ oil as a placebo daily for 90 days. No statistically significant differences between the active and placebo groups were observed, probably due to the small number of participants. However, in the combined fish and flaxseed oil group there was a trend towards increased tear secretion as measured by the Schirmer test and by fluorophotometry. The symptoms of dry eye were reduced during the intervention in both groups. The positive effect was more pronounced in the active group: 70% and 37% of the symptomatic patients became asymptomatic in the active and placebo groups, respectively. The tear film stability and the lipid composition of the meibum were unaffected by the oil supplementation (*30*).

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Sea Buckthorn Oil

Sea buckthorn (*Hippophaë rhamnoides*) is a traditional health-promoting plant that is nowadays cultivated for food in Europe, in several Asian countries, and in the USA and Canada. The berries are rich in phenolic compounds, vitamin C and berry oil (*31–33*). Intake of a low dose of sea buckthorn berries for 3 months reduced the circulating levels of the inflammatory marker C-reactive protein in healthy adults (*34*). The oil content of sea buckthorn seeds is commonly around 10%. The main fatty acids of the berry oil are palmitoleic, palmitic and oleic acids, whereas the seed oils contain mostly linoleic, α-linolenic and oleic acids. The berry oil is especially rich in carotenoids, and both oils have high concentrations of tocopherols, tocotrienols and phytosterols (*35–37*).

Animal studies indicate that sea buckthorn oil promotes wound healing, has cytoprotective and antioxidative effects and may protect against gastric ulcers (*38–41*). Beneficial effects of sea buckthorn oils on atopic dermatitis and mucous membrane have been observed in humans, as well as anti-aggregatory activity (*42–44*).

Recently, positive effects of sea buckthorn oil on dry eye were observed in a double-blind, placebo-controlled, parallel study of 100 volunteers carried out at the University of Turku, Finland (*45*, *46*). During the intervention period of three months the participants consumed 2 g of sea buckthorn or placebo oil daily in the form of capsules. Clinical dry eye tests and symptom follow-ups were performed at the beginning of the intervention, at one month and at three months. A post-check was scheduled one to two months after the intervention. A total of 86 participants completed the study (n=45 in the sea buckthorn group; n = 41 in the placebo group).

At each study visit several dry eye tests were performed. The tear film osmolarity (mOsm/L) was measured using an electrochemical osmolarity meter (TearLabTM, OcuSense Inc., San Diego, CA). Tear film stability was measured as tear film break-up time (TBUT) and tear secretion was analyzed using the

Schirmer test without anesthesia. The fatty acid composition of the tear film was analysed using samples collected on Schirmer papers and gas chromatography. The participants answered a modified version of the validated dry eye symptom questionnaire (the Ocular Surface Disease Index; mOSDI) at each visit.

During the intervention the participants kept a daily logbook concerning their dry eye symptoms. The common symptoms were listed and the participants were asked to assess the severity of the symptoms using a scale from 0 = no symtoms to 3 = severe symptoms. The participants were asked to daily report in the symptom logbooks whether they had taken the study capsules. Participants taking the sea buckthorn/placebo capsules for at least 80% of the intervention days were considered compliant (n = 81).

The sea buckthorn oil used in the study was combined oil form the seeds and berries of sea buckthorn. It was extracted using a supercritical carbon dioxide process by Aromtech Ltd. (Tornio, Finland). The main fatty acids in the oil were palmitoleic (22% w/w), palmitic (22%), oleic (21%), linoleic (16%) and α-linolenic (10%) acids. The daily dose of sea buckthorn oil contained 1.8 mg of carotenoids, 6.0 mg of α-tocopherol, and 0.8 mg of γ-tocopherol. The placebo was a mixture of medium chain triacylglycerol fraction from palm and coconut oils.

The intervention lasted from autumn to winter. There was a considerable drop in the mean temperature in Turku from +8 °C at the beginning of the study to −5 °C at the end of the intervention. During the cold months, the air humidity was low indoors and outdoors. Low relative humidity increases tear evaporation, and dry eye symptoms are more common during cold periods (*47*, *48*). For this reason, there was a general increase in tear film osmolarity both in the sea buckthorn group and in the placebo group. However, the increase from baseline to the end of the intervention was significantly lower in the sea buckthorn group (an increase of 8 and 12 mOsml/L in the sea buckthorn and placebo groups, respectively; P = 0.04, all participants included; P = 0.02 when only the compliant participants were included).

Of the symptoms assessed in the logbooks, redness and burning of eyes were less severe in the sea buckthorn group compared to the placebo. The difference between the groups was significant for redness when all participants were included (P = 0.04). A positive trend was seen when only the compliant participants, consuming the study products for at least 80% of the intervention days, were included (P = 0.11). For burning, the result was significant in compliant participants (P = 0.04) and a positive trend was observed when all participants were included in the analyses (P = 0.05). In the subgroup of contact lens wearers a positive effect of sea buckthorn oil on the frequency of general, non-specified eye symptoms was observed in all participants (P = 0.049), but not when only the compliant participants were included (P = 0.19). Intake of sea buckthorn oil did not affect tear secretion (Schirmer test), the stability of the tear film (TBUT), the symptom scores of the mOSDI questionnaire or other individual symptoms assessed in the symptom logbooks (*45*).

Sea buckthorn oil supplementation did not affect the fatty acid composition of the tear film (*46*). This observation was similar to that of Wojtowicz et al. (*30*), who reported the beneficial effects of n-3 supplementation on dry eye but did not

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however observe changes in the lipid composition of meibum. The effects of sea buckthorn oil are likely to be mediated *via* modulation of the inflammation associated with the dry eye. In addition to the fatty acids, other components of sea buckthorn oil may have contributed to the effect. Carotenoids, tocopherols and tocotrienols have anti-inflammatory and antioxidative properties (*14*, *49*). Several carotenoids in sea buckthorn oil have provitamin A activity (*50*), and may therefore affect the differentiation of the cornea and conjuctiva cells. Effects on the differentiation of meibocytes by the sea buckthorn oil fatty acids are possible (*51*).

Summary

In summary, beneficial effects of antioxidant supplementation on dry eye have been reported in humans. The combination of linoleic and γ-linolenic acids positively affected the signs and symptoms of dry eye in several studies of different populations. Epidemiological investigations and interventions indicate the benefits of n-3 fatty acids. Combined sea buckthorn seed and berry oil is rich in lipophilic antioxidants, linoleic and α-linolenic acids. Positive effects of sea buckthorn oil on dry eye are likely to be mediated via effects on inflammation and oxidative damage.

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Chapter 31

Citrus Monoterpenes: Potential Source of Phytochemicals for Cancer Prevention

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Dietary monoterpenes are non-nutritive volatile principles found in citrus and other regularly consumed plant constituents. Monoterpenes are gaining significant importance as major ingredients of functional food and constituents of prophylactic formulations due to their ability to stabilize the symptoms of chronic diseases including cancer. Among the monoterpenes in citrus, D-limonene is the most common bioactive compound ranging from 30-96% of total volatile oil. The citrus monoterpenes have shown inhibition of human cancer cells proliferation and tumors growth through various mechanisms such as, induction of detoxifying phase-II enzymes, altering the genes responsible for activation apoptosis, inhibition of growth proteins, cell cycle arrest and inhibition of hormonal activities. The advantage of D-limonene is that the metabolites are also found to be effective inhibitors of cancer cells. The major active metabolites of D-limonene, perillyl alcohol, perillic acid and limonene 1, 2-diol are known for the inhibition of human colon, breast and prostate cancer cells. Our research has demonstrated that volatile oil isolated from *Citrus aurantifolia* and *C. senesis* (rich in D-limonene and D-dihydrocarvone) is capable of inhibiting proliferation of human colon cancer cells by inducing apoptosis and inhibition of angiogenesis, a potential target for cancer chemoprevention. In this chapter, health attributes of dietary monoterpenes and their role in

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chemoprevention of cancer is discussed with emphasis on citrus monoterpene D-limonene.

Keywords: Apoptosis; colon cancer; citrus; essential oil; monoterpenes

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Introduction

Monoterpenes mainly present in volatile oils are used widely in food and pharmaceutical industry. They are also referred as ‘essential oils’ due their fragrance/essence. According to United Nation’s COMTRADE data base, global imports of essential oil was \$2 billion in 2005. USA, France and UK were top three producers of the global essential oil (http://comtrade.un.org). Data from Caribbean community suggest that essential oil of orange constituted 10% of the total trade (www.caricom.org). More than 3000 essential oils are known and approximately 300 are widely used for different purposes. The world demand for essential oils, herbal extracts and natural fragrance blend is expected to increase by 4.3% by 2014, amounting to approximately \$23.5 billion. World flavor and fragrance demand was more than \$ 19 billion in 2009, of which 29% was from North America and 28% was from Western Europe. Recently, India and China have emerged as potential exporters of essential oils for future (*1*).

Some of the commonly used essential oils of citrus species are from sweet orange (*Citrus sinensis*), bitter orange (*Citrus aurantium* L), lemon (*Citrus limon*), lime (*Citrus aurantifolia*), grapefruit (*Citrus paradisi*), bergamot (*Citrus bergamia*), mandarin (*Citrus reticulata* Blanco), tangerine (*Citrus reticulata*), and neroli (*Citrus aurantium var. amara* or *bigaradia*).

Chemistry of Citrus Monoterpenes

Monoterpenes are class of terpenoids, which consist of two isoprene units with general molecular formula $C_{10}H_{16}$. These occur as cyclic or linear molecules and most of the monoterpenes are formed through rearrangements and oxidation of the original molecule. Majority of the monoterpene are secondary products from plants and only few are produced in animals and microorganisms (*2*). The monoterpenes are synthesized from geranyl pyrophosphate through oxidation, cyclization or rearrangement. Chemical structures of some of the biologically active dietary monoterpenes are shown in Figure 1A.

Extraction Techniques and Analysis of Monoterpenes from Citrus

Extraction techniques and analysis of monoterpenes from citrus In laboratory and pilot scale citrus monoterpenes can be extracted by either by hydro-distillation techniques using Clevenger’s type apparatus, or Nickerson apparatus and supercritical extraction (*3–6*). Steam distillation is most commonly used in industries for production of volatile oil (*6*). The chemical composition of volatile oils will be analyzed by Gas chromatography. In addition, NIR (near infrared)-spectroscopy was also used to analyze commercial citrus oils (*7*). Other techniques used for quantitative analysis of citrus oil are ATR/FT-IR (Attenuated Total Reflectance/ Fourier transform infrared) and NIR-FT Raman Spectroscopy (*8*).

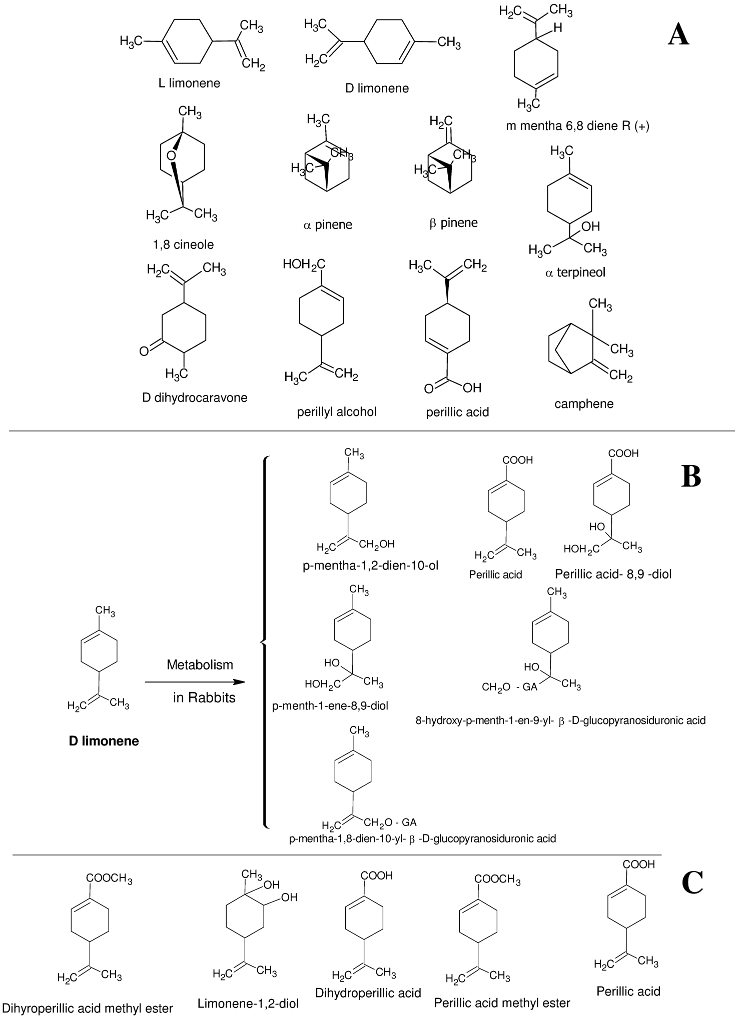
A
L limonene
D limonene
m mentha 6,8 diene R (+)
1,8 cineole
α pinene
β pinene
α terpineol
D dihydrocaravone
perillyl alcohol
perillic acid
camphene
B
D limonene
Metabolism
in Rabbits
p-mentha-1,2-dien-10-ol
Perillic acid
Perillic acid- 8,9 -diol
p-menth-1-ene-8,9-diol
8-hydroxy-p-menth-1-en-9-yl- β -D-glucopyranosiduronic acid
p-mentha-1,8-dien-10-yl- β -D-glucopyranosiduronic acid
C
Dihyroperillic acid methyl ester
Limonene-1,2-diol
Dihydroperillic acid
Perillic acid methyl ester
Perillic acid

Figure 1. A. Chemical structure of biologically active monoterpenes found in diet. B. Metabolites of D-limonene identified in rabbit (58) and C. Major D-limonene metabolites identified in human serum (59).

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Biological Activities of Monoterpenes

Monoterpenes are known for number of biological activities, including antimicrobial and anti-fungal activity, enhancement of skin health, anti-inflammatory activity, analgesic and properties of inhibition of cancer cells proliferation (*9–12*). The volatile monoterpenes are clinically used for pulmonary obstruction and acute bronchitis (*13*) . Numbers of monoterpenes rich volatile oils are explored for health benefits using *in vivo* and *in vitro* models, however very few are clinically studied. Some of the volatile oils clinically tested for their health benefits include, 1,8- cineole, standardized myrtol (mixture of 1,8- cineole, α-pinene and limonene), thyme oil and peppermint oil (*14*). Another major therapeutic area which utilizes monoterpene rich volatile oil is 'aromatherapy'. It is most actively growing system of alternative medicine, which combines massage, counseling and use of plant aromas to treat various ailments (*15*). Some of the clinical investigations conducted on aromatherapy includes, use of lavender oil in peritoneal repair after child birth (*16*), lavender foot massage in intensive care unit (*17*), use of ylang and other oil in epileptic patients (*18*). These studies suggest that monoterpenes are capable of providing a wide range of health benefits. Monoterpenes are easily absorbed by the body and can cross the blood brain barrier efficiently (*19*). Therefore, the common effect of volatile oils are CNS mediated, sedation and stimulation (*15*). The most commonly used essential oil and their pharmacological actions are listed in Table 1.

Table 1. Pharmacological activity of monoterpenes rich essential oils

Condition	***Most commonly used volatile oil rich in monoterpenes***	***Reference***
Sedative	Chamomile, Bergamot, Geranium, Lavender, Lemon, Marjoram, Rosewood, Sandalwood, Valerian	Referred in (*15*)
Stimulant	Basil, Jasmine, Chamomile, Patchouli, Peppermint, Ylang ylang	Referred in (*15*)
Anti-inflammatory	Eucalyptus Black cumin seed *Lavandula angustifolia* Mill. Tea tree oil Lippa multiflora *Cymbopogon citrates* *Satureja hortensis* L	(*60*) (*61*) (*62*) (*63*) (*64*) (*65*) (*66*)
Antipyretic	Lippa multiflora *Calamintha sylvatica* subsp. *Artemisia caerulescens* subsp. *gallica*	(*64*) (*67*) (*68*)
Induction of Anastasia in children	Sweet orange	(*69*)
Diuretic	Anthemis nobilis	(*70*)

Continued on next page.

Table 1. (Continued). Pharmacological activity of monoterpenes rich essential oils

Condition	*Most commonly used volatile oil rich in monoterpenes*	*Reference*
Lipid lowering	lemongrass oil	(*71*)
Anthelmienthic	*Ocimum gratissimum* Linn.	(*72*)
Antispasmodic and anti-diarrhoeal	Satureja hortensis L	(*73*)
Anti stress	Lemon oil	(*74*, *75*)
Anti-conversant	Clove (*Eugenia caryophyllata*)	(*76*)
Antileishmania	*Ocimum gratissimum*	(*77*)
Hypotensive	Croton nepetaefolius, *Mentha x villosa*	(*78*, *79*)

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The monoterpenes from citrus are well known for their antimicrobial activity against wide range of organisms, including *Helicobacter pylori* (*20*, *21*). Additionally, they have also demonstrated sedative, anti-stress, antidepressant activity, ability to potentiate the activity of anesthesia in children, (*22*) , anti-inflammatory (*23*) and mild aphrodisiac activity (*24*). Other health benefits claimed by the sellers of these products based on the non referred information include, antispasmodic, antiseptic, carminative, diuretic, cholagogue activity (*25*). One of the major monoterpene D-limonene, which is abundantly found in citrus species, has clinical significance for colon and breast cancer prevention (*26*).

Therapeutic Benefits of D-Limonene from Citrus

The advantages of D-limonene are fast absorption and production of biologically active metabolites (*27*). Structure of the selected metabolites observed from rabbit urine and feces are shown in Figure 1 B and similar metabolites were also found in a human study. The LD_{50} value for D-limonene in male and female mice is 5.6 and 6.6g/kg body weight, respectively. The same is 4.4 and 5.1 g/kg body weight in case of male and female rats (*28*). The research from national toxicology program (NTP) has shown that administration of different doses (413-6,600 mg/kg) of D-limonene daily for five days/week for three weeks did not result in any signs of toxicity upto the dose of 1,650 mg/kg (*29*). In humans, except for increase in bowel movements, no abnormal or toxic symptoms were observed based on blood test, liver, pancreas and kidney test with administration of single dose of 20 g D-limonene (*30*). Based on the clinical investigation dose equivalent to 7.0 g of D-limonene was found to be safe for an average adult weighing 60 kg (*27*). D-limonene is listed in the code of federal regulations to be generally recognized as safe (GRAS) to be used as flavoring agent in food. Based on the information available the average intake of D-limonene by American is 16.2 mg/person/day (Avg. 60 kg person) (*31*). The advantages with D-limonene is the fast and complete absorption by oral and other routes of administration, rapid metabolism and distribution in different organs (*32*). D-limonene and its metabolites are detected in serum, lung, liver, kidney as well as relatively higher concentrations are found in adipose tissues, including mammary of rats (*33*, *34*). The half life of D-limonene in human is 12 -24 h and a major part is excreted through kidney (*28*). The major urinary metabolites of D-limonene in human are glucuronides of perillic acid, dihydroperillic acid, limonene-8,9-diol, and monohydroxylated limonene, perillic acid (*27*, *32*). Some of the metabolites of D-limonene analyzed in human serum are as shown in Figure 1C.

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Mode of Cancer Cells and Tumor Inhibition by Monoterpenes

D-limonene constitutes a major compound (30-96%) in most of the citrus species. Minor monoterpenes are β-pinene, γ-terpene, α-pinene, myrcene and linalool representing less than 15% of total monoterpenes. Research on cancer prevention is focused mainly on D-limonene and its active metabolites, perillic acid and perillyl alcohol. Citrus monoterpenes have shown inhibition of lung, colon, liver, leukemia, mammary cancer cells proliferation and inhibition of chemically induced rodent mammary and pancreas carcinoma (*3*, *35–37*). Number of research reports explaining the proliferation inhibition and tumor inhibition ability of monoterpenes from citrus was documented. Additionally these compounds have also shown inhibition of cancer cells through unique mechanisms such as, post translational isoprenylation of growth controlling *Ras* oncoproteins in pancreatic cancer cells (*38*). These *Ras* proteins are also known as small molecular weight G-proteins, which regulate survival and growth of the cells, whose dysregulation is known to cause cancer. Carvacrol, a principle monoterpene of many dietary components showed inhibition of human non-small cell lung cancer (NSCLC) cell line, A549 (*39*). Transgenic mouse embryonic NIH 3T3 fibroblast cells trasfected with D-limonene synthase gene from Japanese catnip (*Schizonepeta tenuifolia*) has shown induction of apoptosis in limonene producing cells. Induction of apoptosis in these cells were characterized by increase in apoptosis related Bcl-2 family proteins and decrease in the level of Bad and phosphorylated JNK (*40*). Menthol, a monoterpene found in mint and other plants has shown inhibition of 7,12-dimethylbenz (a) anthracene (DMBA)-induced mammary rat cancer when supplemented as 1% of daily diet (*41*). Menthol has also shown induction of apoptosis in human bladder cell lines T24 through inhibition of transient receptor potential melastatin 8 (TRPM8)-dependent pathway through alteration of intracellular calcium (*42*)

Carvacrol from *Origanum onites L.* demonstrated inhibition of DNA synthesis in *N-ras* transformed mouse myoblast cells, CO25 (*43*). Calcium dependant inhibition of NFκB by perillyl alcohol was observed in WEHI-231 B-lymphoma cells (*44*). Phosphorylation of BRCA1 (breast cancer–associated gene 1) by a bicyclic monoterpene diol was observed in human keratinocytes and this phosphorylation is helpful in protection of cells against UNB-induced damages (*45*). Volatile oil from *Schefflera heptaphylla* (L.) rich in β-pinene has been demonstrated to inhibit human skin, hepatic and breast cancer cells proliferation with IC_{50} values of 147.1 to 264.7 μM (*46*).

Induction of phase-II enzymes, initiation of apoptosis, inhibition of post translational isoprenylation of cell growth-regulating proteins seems to play a major role in cancer inhibition by D-limonene and perillyl alcohol (*26*). Limonene also demonstrated antioxidant activity and protection of cells against oxidative stress by inhibiting H_2O_2 via increased activity of catalase and peroxidase enzymes (*47*). Ascaridole a monoterpene found in *Croton regelianus* Muell. Arg., a native plant of Brazil has shown inhibition of different cancer cells effectively with IC_{50} values of 4.2-23.7 ppm and inhibited mouse Sarcoma 180 Tumor at 50 and 100 mg/kg (*48*).

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Citrus Monoterpenes and Colon Cancer

Dietary perillyl alcohol and D-limonene have shown inhibition of azoxymethane (AOM) induced colon tumor at dose of approximately 1g/ kg body weight (*49*). Induction of apoptosis was also observed in the colon of these animals. D-limonene also showed similar effect on azoxymethane (AOM) induced F344 rat model. Treatment of 0.5% D-limonene in drinking water for a week before administration of AOM has resulted in significant ($P<0.001$) decrease in the frequencies of ACF and crypts/colon and aberrant crypts/focus compared to control animals (*50*).

Inhibition of cell cycle at G1 phase by perillyl alcohol and perillic acid was reported based on their ability to up regulate expression of cdk inhibitor $p21^{Waf1/Cip1}$, cyclin E and down-regulate of cyclin D1, cyclin-dependent kinase (cdk) 4 and cdk2 expressions (*51*).

Auraptene, a citrus volatile oil inhibited formation of ACF in AOM challenged F344 male rats. Auraptene exhibited dose dependent inhibition of ACF at 100 and 500 ppm for 5 weeks with diet (*52*). Phase-II trail of perillyl alcohol on patients with metastatic colon cancer suggested that oral administration has no clinical antitumor activity in the patients with advanced colon cancer (*53*).

D-limonene is referred to as one of the most effective natural compounds against colon cancer in animal model (*54*). It has suppressed murine colon tumor through inhibition of cholesterol synthesis and hepatic metastasis (*55*). Inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced neoplasia of the lungs and forestomach was also observed with administration of D-limonene prior to carcinogen in female A/J mice (*35*). Administration of 1% D-limonene at the dose of 15 ml/kg, every other day for seven weeks reduced gastric tumor in orthotropic nude mice model. Results of this study also indicated significant decrease in micro vascular density and expression of VEGF with D-limonene treatment. Furthermore, there was a synergistic inhibition of tumor and other markers such as, VEGF, apoptotic index and micro vessel density in animal treated with combinations of D-limonene with 5-fluoruracil (30 mg/kg/day), a chemotherapeutic drug (*56*). D-limonene is known to elevate the activity of GST, GST isoenzyme level, GSH and glutathione peroxidase (GPx) when supplemented in diet at 10,000 ppm for two weeks in experimental rats (*57*).

Results of this study suggest the ability of D-limonene to act on phase-II enzymes, which help in detoxication, thus preventing various types of cancer.

Recently, our studies demonstrated that monoterpenes from *Citrus aurantifolia* (key lime) inhibited human colon cancer (SW480) cell proliferation. Total volatile oil was isolated from key lime using Clevenger apparatus and 22 compounds were identified by GC-MS analysis, which constitute more than 89% of the volatile components. D-limonene and d- dihydrocarvone are the major compounds (constituting more than 60% of total volatile principles). Other major monoterpenes identified were m-Mentha-6, 8-diene, α-terpineol, β-linalool and *trans* bergamotene. The volatile oil has demonstrated dose and time dependant inhibition of SW480 cell proliferation. Interestingly, the volatile oil was not toxic to mouse embryonic fibroblast (NIH3T3) cells. Furthermore, induction of caspase-3 mediated apoptosis was found to be the possible cause for proliferation inhibition (*3*). Our recent research on volatile oil from other citrus species, which is rich in D-limonene (>90% of total monoterpenes) has shown differential inhibition of cancer cell proliferation (pancreatic, colon, breast and prostate). Based on the current knowledge on cancer chemoprevention, major targets of D-limonene to inhibit tumor and proliferation are summarized in Figure 2.

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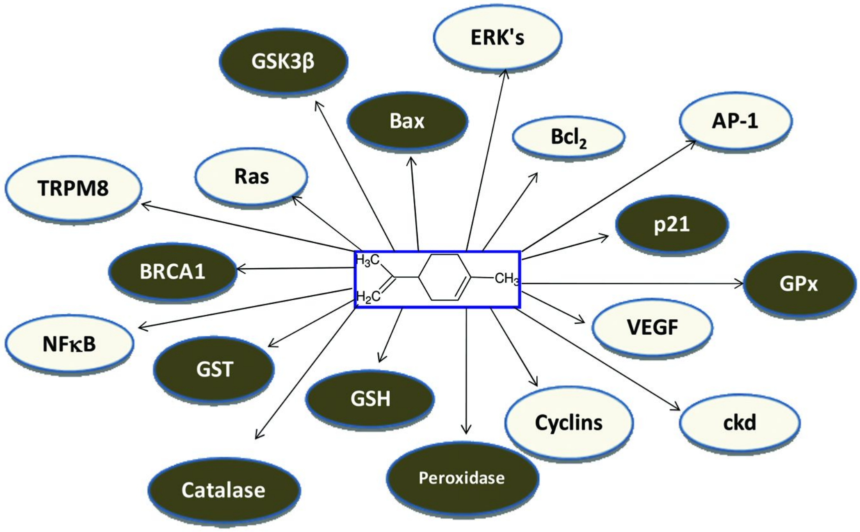

Figure 2. Major cancer related genes and enzymes targeted by D-limonene. The dark circle represents up-regulation/elevation of the activity or phosphorylation and white circle represents down-regulation.

In summary, D-limonene, a major monoterpene of citrus, is known for several health benefits including chemoprevention of cancer. D-limonene and its major metabolites such as, perillic acid, perillyl alcohol seems to act on various genes specific to cancer environment to prevent cell proliferation, tumor growth and metastasis. Some of the targets include induction of phase-II enzymes, cell cycle arrest, induction of apoptosis, inhibition of metastasis, anti-inflammatory, anti-angiogenesis and induction of antioxidant enzymes. D-limonene is listed as a GRAS agent, suggesting its non-toxic nature to normal cells. Therefore, D-limonene a major constituent of citrus volatile oil may serve as a potential chemopreventive agent for cancer of the colon and other organs.

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Chapter 32

Anthocyanin Bioavailability: Past Progress and Current Challenges

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Anthocyanins are the red, blue, and purple pigments present throughout nature. Foods rich in anthocyanins include berries, red cabbage, radish, eggplant, blue corn, and purple carrots, as well as many other red, purple, and blue fruits, vegetables, and legumes. Evidence continues to accumulate suggesting multiple roles for dietary anthocyanins in promoting health and preventing disease. Anthocyanins have been associated with reduced risk for cardiovascular disease, cancer, diabetes, and cognitive decline. All of these beneficial health effects require that the anthocyanins be well-absorbed. However, bioavailability studies have suggested otherwise. New evidence is emerging that may begin to explain this incongruity.

Introduction

Anthocyanins and Health

Studies with both animals and humans suggest that anthocyanin-rich products reduce risk of cardiovascular disease. In animal studies, anthocyanin-rich products have been shown to protect heart tissue from ischemic insult (*1*) and to reduce aterial plaques (*2*, *3*). Human intervention trials have shown anthocyanin-rich foods or supplements decrease lipid hydroperoxides (*4*), decrease LDL cholesterol (*5*, *6*), increase HDL cholesterol (*7*), and reduce systolic blood pressure (*7*).

Berries, which are very high in anthocyanins, have been shown to interfere with carcinogenesis. For example, extracts from blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry inhibited growth of human neoplastic cell lines of oral, breast, colon, and prostate tissue (*8*). In animal

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studies, berry products have been shown to interfere with carcinogenesis in models of colon (*9*) and esophageal cancer (*10*, *11*). A clinical study of patients with the precancerous condition called Barrett's esophagus, black raspberry administration decreased oxidative stress and DNA damage (*12*).

Cognitive function also appears to be improved by anthocyanin intake. Rats fed blueberry supplements for 8 weeks showed improved performance on a spatial memory test, and performance was correlated to anthocyanin accumulation in portions of the brain related to memory (*13*). Similarly, older adults consuming blueberries for 12 weeks had showed improvements in memory (*14*). Pomegranate juice fed to pregnant dams protected brain tissue of pups from hypoxia-ralated damage during delivery (*15*).

Anthocyanin preparations also seem to counter obesity and diabetes. Mice fed anthocyanin preparations with a high fat diet had lower body weight and percent body fat than those fed a high fat diet without the anthocyanin-rich preparations (*16–19*), and certain anthocyanin-rich preparations improved glycemic control in mice fed high fat diets (*16–20*). This effect may be partly mediated though stimulation of insulin secretion by pancreatic β-cells (*21*).

Both animal and human studies suggest important biological actions of anthocyanins *in vivo* with respect to many chronic diseases, including cardiovascular disease, cancer, diabetes, and age-related cognitive decline and brain function. For anthocyanins to exhibit biological effects on these processes, they must reach the systemic circulation in biologically meaningful quantities. However, a clear understanding of anthocyanin bioavailability has been elusive, as there is a consistent discrepancy between the apparent bioactivity of anthocyanins and what we've been able to observe with respect to their bioavailability.

Anthocyanin Bioavailability: Past Progress

Animal Studies of Anthocyanin Bioavailability

Animal studies of anthocyanin bioavailability have been conducted primarily in rats, with additional studies in pigs and rabbits (*22–39*). Doses in these studies have been fairly large (several hundred mg per kg body weight, whereas a single large serving of an anthocyanin-rich food might deliver several hundred mg total). Earliest studies were conducted with bilberry, elderberry, or black currant, as these are extremely rich sources of anthocyanins (*40*). Other sources have included purple corn, blackcurrant, purple rice, chokeberry, and raspberry. These studies (*22–31*, *41–45*) have consistently shown a number of phenomena, including (i) peak plasma response after the dose occurs very quickly, usually within the first two hours, (ii) peak plasma concentrations are low, usually less than one micromolar, and (iii) recovery of anthocyanins in urine are typically less than one percent. In most of these early animal studies, only anthocyanin glycosides were observed in plasma and urine, while no aglycones were detected. From these studies it was concluded that anthocyanins are absorbed mainly intact and reach peak plasma concentration very quickly.

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Human Studies of Anthocyanin Bioavailability

Human bioavailability studies with anthocyanins have confirmed findings with animals (*25*, *42*, *46–62*). The studies with humans have used large but more realistic doses of several hundred milligrams, which could be achieved with a single large serving of berries for example, though some studies have exceeded a gram. Some of these studies have been performed with extracts or powders of products like elderberry, blueberry, blackberry, boysenberry, or chokeberry to deliver these large doses of anthocyanins, while others have involved intact foods. As with the animal studies, the time to reach peak plasma concentration has been short, usually less than 2 hours (*25*, *26*, *42*, *47*, *49*, *51*, *54*, *58*, *59*). This is notably faster than peak absorption times for other polyphenols (*63*). Urinary accumulation also occurs fairly quickly, with the fastest rate of movement into urine occurring between 2 and 4 hours after the dose (*59*, *61*). Peak plasma concentrations have been consistently low, generally less than 100 ng/mL, and urinary recovery is typically a fraction of a percent (*25*, *26*, *48–51*, *53–56*, *59*, *61*, *62*). This is also different from recovery of other polyphenols, which is for the most part much higher (*63*). Many studies have shown that anthocyanins can be absorbed intact (*22–31*, *41–45*) , and while the importance of intact anthocyanins compared to metabolic or degradation products remains unclear (*16*, *24*, *64*, *65*), it has been suggested that degradation products may be important for the ultimate bioactivity of dietary anthocyanins (*66*, *67*).

Factors Affecting Absorption of Anthocyanins

Chemical structure is an important determinant of anthocyanin bioavailability. The anthocyanidin backbone is one chemical characteristic that affects bioavailability. Most notably, pelargonidin-based anthocyanins, the primary anthocyanidin in strawberries, appear to be absorbed and metabolized differently than anthocyanins with other anthocyanidin backbones (*29*). First, pelargonidin-based anthocyanins are found mainly as glucuronidated metabolites (*29*, *52*, *60*). Second, recovery of pelargonidin-based anthocyanins (specifically, pelargonidin 3-glucoside) after consumption has been about 2 % (*52*, *60*), whereas anthocyanin recovery from other sources has been a fraction of a percent (*25*, *26*, *48–51*, *53–56*, *59*, *61*, *62*). A study of pigs fed black currant powder suggested that cyanidin-based anthocyanins are more efficiently absorbed than delphinidin-based anthocyanins (*30*), and a study of humans and rats fed a mixture of berry anthocyanins showed that malvidin-based anthocyanins are more efficiently absorbed than delphinidin-based anthocyanins (*51*).

The sugar moiety also influences bioavailability. When weanling pigs were fed black currant powder, it was found that rutinoside recovery in urine was higher than that for glucosides of the same anthocyanidin (*30*). Further, recovery of cyanidin 3-sambubioside and cyanidin 3-sambubioside-5-glucoside were higher than that for cyanidin monoglycosides (*30*). In a study of men consuming freeze-dried black raspberries, absorption efficiency of cyanidin 3-xylosyl rutinoside appeared to be higher than that of cyanidin 3-rutinoside, based on urinary recovery (*68*). When men and women consumed elderberry concentrate, percent recovery of cyanidin 3-sambubioside was greater than that for cyanidin 3-glucoside (*50*).

Acylation is another structural feature that dramatically affects anthocyanin absorption. In bioavailability studies with purple carrot, recovery of acylated anthocyanins was 11 to 14 fold less in urine and 8 to 10 fold less in plasma compared to that of nonacylated anthocyanins (*59*). Similarly, recovery of nonacylated anthocyanins from red cabbage were more than 4-fold that of acylated anthocyanins (*61*). Results reported by Wu et al. (*29*) after pigs consumed blackberries also showed that acylated anthocyanins were less bioavailable than nonacylated anthocyanins.

Due to the polarity of anthocyanins (as well as other flavonoid glycosides), it has been presumed that they would not transfer passively across a lipid bilayer (*69*, *70*). It has been suggested that anthocyanin glycosides may be hydrolyzed to form aglycones by enzymes such as lactase phloridzin hydrolase (*71*, *72*), but since the aglycones have not been recovered in biological samples, this is not a widely supported hypothesis. That said, the aglycones are very unstable near neutral pH (*66*, *73*). Another possible mechanism of absorption may be transport of the glucoside across the small intestine by the sodium-dependent glucose cotransporter (SGLT1) (*74*). Mulleder et al. (*50*) showed that sucrose ingestion with elderberry concentrate resulted in reduced absorption of anthocyanins. Bilitranslocase is another proposed transport protein, and this is supported by the competitive inhibition that anthocyanins exhibit on bilitranslocase activity (*75*).

One possible explanation for the low apparent bioavailability of anthocyanins is the lack of detection of metabolites formed. Early bioavailability studies revealed the formation of metabolites. Methylation of anthocyanins was first reported in 1999 by Miyazawa et al. (*23*) in the liver of rats fed cyanidin 3-glucoside and by Tsuda et al. (*24*) in rat liver and kidney extracts after consumption of cyanidin 3-glucoside. Glucuronidation of anthocyanins was first reported in 2002 by Wu et al. (*56*) after women consumed elderberry concentrate. Later studies confirmed that anthocyanins are metabolized through methylation, sulfation, and glucuronidation (*29*, *30*, *32*, *52*, *53*, *55*, *56*, *60*, *61*, *68*, *76*), though in most cases, the intact forms have been present in plasma and urine in larger quantities than conjugated forms.

Tsuda et al. (*24*) were the first to report protocatechuic acid as a potential metabolite of anthocyanins. Recognizing that cyanidin 3-glucoside could react with peroxyl radicals to ultimately form protocatechuic acid *in vitro*, this group sought to show that this conversion can take place *in vivo*. To this end, rats were fed cyanidin 3-glucoside, and blood and tissues were analyzed at several time points over the next 4 hours. Tsuda et al. (*24*) reported that protocatechuic acid was detected in concentrations 8-fold higher than cyanidin 3-glucoside, and suggested that protocatechuic acid may be a major metabolite of cyanidin. Since that report, several other studies have suggested that protocatechuic acid may be an important metabolite of cyanidin (*77*, *78*), while most studies have not reported observing this compound.

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Anthocyanin Bioavailability: Current Challenges

Current Challenge: Improved Chemical Detection Methods

One challenge that has undoubtedly hindered progress in accurately assessing anthocyanin bioavailability is that in solution anthocyanins exist in equilibrium in a number of molecular forms, including the flavylium cation, the quinoidal base, the hemiketal base, and the chalcone (*79*). The distribution among forms depends on the anthocyanin and on pH (*79*). The distribution of forms affects color, with the relatively stable flavylium cation, which exists at very low pH, tending to be red or occasionally yellow, the quinoidal base being blue or red, and the hemiketal base and chalcone being colorless (*57*). Most physiologic environments are characterized by neutral pH, thus the relatively stable flavylium cation would not be the predominant form present, but rather the hemiacetal base or chalcone would be the primary forms. For bioavailability studies, detection methods are typically based on photodiode array detection of the colored flavylium cation after isolation by HPLC. If in the neutral pH of the physiologic compartments, the anthocyanin has been irreversibly transformed to a colorless form, it will escape detection by current lab methods, thus leading to underestimation of bioavailability. Thus, great opportunity exists to advance understanding of anthocyanin bioavailability through development of robust laboratory methods that could precisely and accurately detect anthocyanin forms other than the flavylium cation.

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Current Challenge: Exhaustive Identification of Metabolites and Degradation Products

Another explanation for the discord between the apparent low bioavailability of anthocyanins and the notable biological effects may be the inappropriate focus on the intact anthocyanins and their conjugated forms in biological samples. Alternatively, degradation products and small metabolites may be responsible for the physiologic action of dietary anthocyanins. Deglycosylation of a natural anthocyanin form will produce an unstable aglycone, which at near neutral pH will spontaneously degrade to phloroglucinaldehyde and a phenolic acid (*66*, *73*). The phenolic acids formed upon degradation of cyanidin, pelargonidin, and delphinidin are protocatechuic acid, 4-hydroxybenzoic acid, and gallic acid, respectively (*73*). *In vitro*, Kay et al. (*66*) found that protocatechuic acid and phloroglucinaldehyde can be metabolized to glucuronide and sulphate conjugates, thus this may also be happening *in vivo*.

Anthocyanins that reach the colon intact may be converted to phenolic acids by intestinal microbes. A study with ileostomy patients who consumed raspberries revealed that 40% of the anthocyanins consumed were recovered in the ileal fluid (*80*), and a study with ileostomy patients who consumed blueberries revealed that up to 85% of anthocyanins consumed were recovered in the ileal fluid (*81*), suggesting that a large portion of intact anthocyanins enter the colon for possible degradation by colonic microflora. *In vitro* studies with human colonic microbiota have supported the possibility that these microorganisms transform intact anthocyanins to phenolic acids (*67*, *82*).

A few animal and human studies have reported observation of phenolic acids after consumption of anthocyanins (*24*, *65*), further supporting the potential importance of phenolic acids as bioactive products of anthocyanin consumption. Protocatechuic acid, the primary degradation product of cyanidin, which is the most common anthocyanidin in the food supply, has been shown to have numerous biological effects, including effects related to cardiovascular disease (*83*), inhibition of tumor metastasis (*84*), promotion of apoptosis in multiple neoplastic cell lines (*85*), reduction of inflammation (*86*), and antihyperglycemic and antihyperlipidemic effects on streptozoticin-diabetic rats (*87*, *88*). Gallic acid has been shown to have anti-metastasis effects on gastric cancer cells (*89*).

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Conclusion

A clear understanding of anthocyanin bioavailability has been elusive. Many studies have suggested that the bioavailability of anthocyanins is very low, yet the apparent bioactivity of anthocyanins *in vivo* suggest otherwise. New evidence is emerging to support the prospect that metabolites and degradation products may be the primary forms of anthocyanins absorbed and exhibiting biological activities. Promising opportunities exist for development of better laboratory methods for identifying forms of anthocyanins in plasma, urine, and tissues, and these new methods will likely open the door to a much better understanding of the role of anthocyanins in human health.

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Editors' Biographies

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Dr. Bhimu Patil is the Professor and Director of Vegetable and Fruit Improvement Center. He has published more than 100 research papers and has received 15 awards. Patil and colleagues initiated an international symposium "FAVHealth" symposium in 2005, and hosted in Houston 2007. He has chaired or co-chaired 25 symposiums. He was the invited speaker, including plenary speaker, for his scientific research and educational excellence by several countries including China, South Korea, Brazil, Indonesia, Sweden, France, India, Canada, Portugal, and different states in the U.S.A. He has developed two multidisciplinary and multistate first-of-its kind course, "Science of Foods for Health" and "Phytochemicals in Fruits and Vegetables to Improve Human Human Health".

Guddadarangavvanahally K. Jayaprakasha

G. K. Jayaprakasha has 22 years of research and 3 years of industrial experience in "Chemistry of Natural Products". His research involves bioassay directed discovery, purification and chemical characterization of natural compounds from fruits, vegetables, spices and herbs using preliminary in vitro assays such as anticancer, antimicrobial and antioxidant properties. Isolated and characterized >80 novel, rare and bioactive compounds from Citrus, Pomegranate, Carrots, Cinnamon, Lichen, Turmeric and Garcinia. He filed 24 patents in the U.S., Europe and India. Dr. Jayaprakasha published 120 research papers and 130 presentations in national and international meetings. Based on his research accomplishments, he has been admitted as "Fellow of Royal Society of Chemistry" (FRSC), Royal Society of Chemistry, Cambridge, England.

Kotamballi N. Chidambara Murthy

Kotamballi N. Chidambara Murthy is working as a senior research scientist at Triesta, R&D unit of HCG oncology hospital, Bangalore, India. He is involved in the research on clinical and molecular nutrition with emphasis on cancer from the past 10 years. Dr. Murthy obtained his Masters in Pharmacognosy and Ph.D. in Biotechnology from Central Food Technological Research Institute, India. He has worked as a postdoctoral fellow at Texas A&M University. Dr. Murthy has published over 50 original research papers, 6 reviews, edited two books, contributed to more than 12 book chapters, authored 6 scientific popular articles, presented more than 55 papers at national and International scientific meetings, and delivered over 30 invited talks. He has U.S., European, and Indian patents to

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ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

his credit. He is the recipient of several meritorious awards and fellowships from Indian and International organizations.

Navindra P. Seeram

Navindra P. Seeram, Ph.D., is an Assistant Professor in the Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, U.S.A. Prior to this, he was the Assistant Director of the UCLA Center for Human Nutrition in the Department of Medicine, University of California, Los Angeles, and an Assistant Adjunct Professor in the UCLA School of Medicine. His laboratory investigates plant natural products for therapeutic and preventive effects against cancer and diabetes. Dr. Seeram has co-authored more than 100 peer-reviewed research articles, 14 book chapters, and 6 international patents. He has co-edited 3 books and is the series editor of Clinical Pharmacognosy published by CRC Press/Taylor and Francis. He serves on the editorial boards of the Journal of Berry Research and the International Journal of Applied Research in Natural Products. He was the recipient of the 2009 Young Scientist Award from the Division of Agricultural and Food Chemistry of the American Chemical Society and is regularly quoted in the popular press about the medicinal properties of plant foods. Dr. Seeram did his doctoral and postdoctoral studies at the University of the West Indies in Jamaica and at Michigan State University, U.S.A. respectively.

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APPENDIX A

Chromatographic Techniques for the Separation of Polymethoxyflavones from Citrus (Chapter 1)

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ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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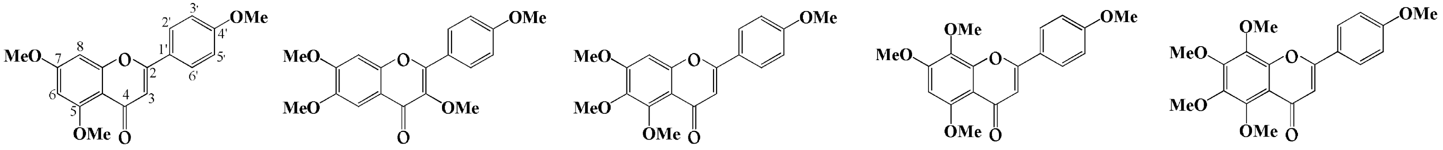
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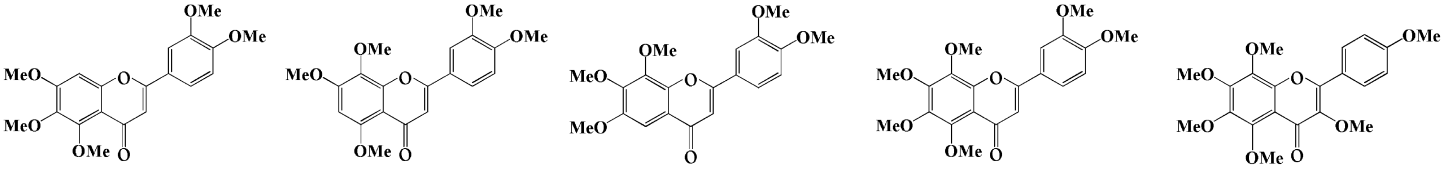
(1) 5, 7, 4’ Trimethoxyflavone

(2) 3, 6, 7, 4’ Tetramethoxyflavone

(3) 5, 6, 7, 4’ Tetramethoxyflavone

(4) 5, 7, 8, 4’ Tetramethoxyflavone

(5) 5, 6, 7, 8, 4’ Pentamethoxyflavone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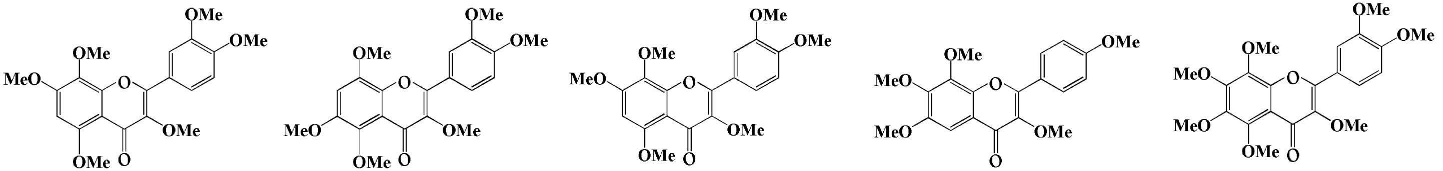
(6) 5, 6, 7, 3’, 4’ Pentamethoxyflavone

(7) 5, 7, 8, 3’, 4’ Pentamethoxyflavone

(8) 6, 7, 8, 3’, 4’ Pentamethoxyflavone

(9) 5, 6, 7, 8, 3’, 4’ Hexamethoxyflavone

(10) 3, 5, 6, 7, 8, 4’ Hexamethoxyflavone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(11) 3, 5, 7, 8, 3’, 4’ Hexamethoxyflavone

(12) 3, 5, 6, 8, 3’, 4’ Hexamethoxyflavone

(13) 3, 5, 6, 7, 3’, 4’ Hexamethoxyflavone

(14) 3, 6, 7, 8, 4’ Pentamethoxyflavone

(15) 3, 5, 6, 7, 8, 3’ 4’ Heptamethoxyflavone

Figure 1. Structures of polymethoxyflavones isolated from citrus.

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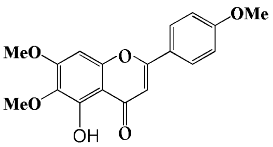
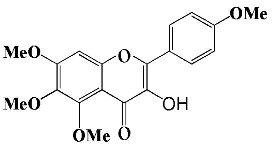
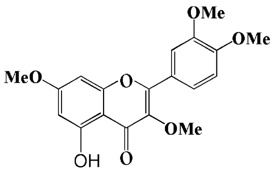


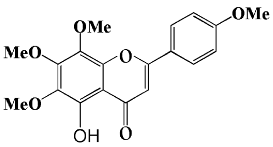
(16) 5- OH- 6,7, 4’
Trimethoxyflavone



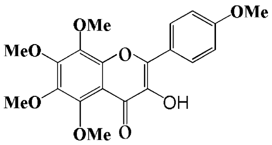
(17) 3- OH- 5, 6, 7, 4’
Tetramethoxyflavone



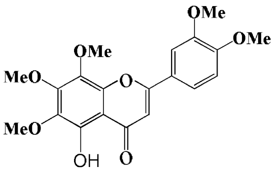
(18) 5- OH- 3, 7, 3’, 4’
Tetramethoxyflavone



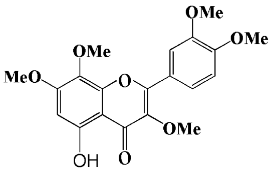
(19) 5- OH- 6,7, 8, 4’
Tetramethoxyflavone



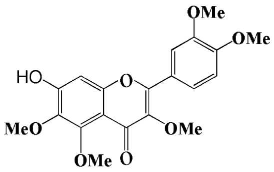
(20) 3- OH- 5, 6, 7, 8, 4’
Pentamethoxyflavone



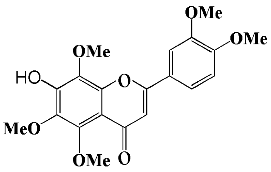
(21) 5- OH- 6, 7, 8, 3’, 4’
Pentamethoxyflavone



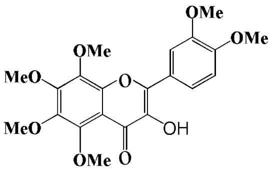
(22) 5- OH- 3, 7, 8, 3’, 4’
Pentamethoxyflavone



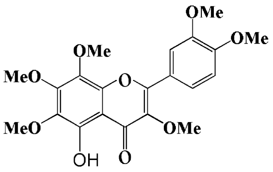
(23) 7- OH- 3, 5, 6, 3’, 4’
Pentamethoxyflavone



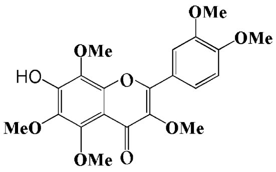
(24) 7- OH- 5, 6, 8, 3’, 4’
Pentamethoxyflavone



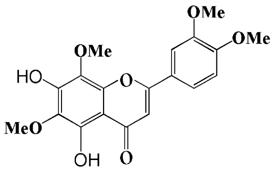
(25) 3- OH- 5, 6, 7, 8, 3’, 4’
Hexamethoxyflavone



(26) 5- OH- 3, 6, 7, 8, 3’, 4’
Hexamethoxyflavone



(27) 7- OH- 3, 5, 6, 8, 3’, 4’
Hexamethoxyflavone



(28) 5,7- OH- 6, 8, 3’, 4’
Tetramethoxyflavone

Figure 2. Structures of hydroxylated polymethoxyflavones isolated from citrus.

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Table 2. ^{1}H NMR of polymethoxyflavones isolated from citrus species

Name[a]	H-3	H-5	H-6	H-8	H-2′	H-3′	H-5′	H-6′	OMe	Ref
5,7,4'- Tri-MF (1)	6.59 s		6.38 (d) (J=2.0)	6.55 (d) (J = 2.0)	7.82 (d) (J = 9.0)	7.00 (d) (J = 9.0)	7.00 (d) (J = 9.0)	7.82 (d) (J = 9.0)	3.82, 3.92, 3.95	*(49)*
3,6,7,4'- Tetra-MF (2)		7.23 s		6.73 s	8.03 (d) (J = 8.4)	7.11 (d) (J = 8.4)	7.11 (d) (J = 8.4)	8.03 (d) (J = 8.4)	3.77, 3.80, 3.95, 3.86	*(27)*
5,6,7,4'- Tetra-MF (3)	6.59 s			6.80 s	7.78 (d) (J = 9.0)	6.97 (d) (J = 9.0)	6.97 (d) (J = 9.0)	7.78 (d) (J = 9.0)	4.10, 3.99, 3.94, 3.88	*(40)*
5,7,8,4'- Tetra-MF (4)	6.60 s		6.44 s		7.90 (d) (J = 9.0)	7.02 (d) (J = 9.0)	7.02 (d) (J = 9.0)	7.90 (d) (J = 9.0)	3.89, 3.96, 3.99, 4.01	*(40)*
5,6,7,8,4'- Penta-MF (*Tangeretin*) (5)	6.60 s				7.88 (d) (J = 9.0)	7.02 (d) (J = 9.0)	7.02 (d) (J = 9.0)	7.88 (d) (J = 9.0)	4.10, 4.02, 3.95, 3.95, 3.89	*(40)*
5,6,7,3',4'- Penta-MF (*Sinensitin*) (6)	6.59 s			6.80 s	7.33 (d) (J = 2.1)		6.98 (d) (J = 8.4)	7.52 (dd) (J = 2.1, 8.4)	4.00, 4.00, 3.98, 3.96, 3.92	*(40)*
5,7,8,3',4'- Penta-MF (7)	6.61 s		6.44 s		7.42 (d) (J = 2.4)		6.98 (d) (J = 2.4)	7.58 (dd) (J = 2.4, 8.4)	4.01, 3.99, 3.97, 3.96, 3.96	*(40)*
6,7,8,3',4'- Penta-MF (8)	6.78 s	7.20 s			7.54 (d) (J = 2.0)		7.11 (d) (J = 8.5)	7.65 (dd) (J = 8.5, 2.0)	3.81, 3.96, 3.77, 3.89, 3.85	*(27)*
5,6,7,8,3',4'- Hexa-MF (*Nobiletin*) (9)	6.61 s				7.40 (d) (J = 2.0)	-	7.00 (d) (J = 8.0)	7.57 (dd) (J = 2.0, 8.0)	3.96, 3.96, 3.97, 3.98, 4.04, 4.10	*(49)*
3,5,6,7,8,4'- Hexa-MF (10)					8.14 (d) (J = 9.0)	7.04 (d) (J = 9.0)	7.04 (d) (J = 9.0)	8.14 (d) (J = 9.0)	4.09, 4.00, 3.97, 3.95, 3.90, 3.87	*(28)*
3,8,5,7,3',4'- Hexa-MF (11)			6.43 s		7.84 (d) (J = 2.0)		7.01 (d) (J = 8.0)	7.86 (dd) (J = 2, 8)	3.90, 3.94, 3.97, 3.97, 4.01, 4.02	*(49)*

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Table 2. (Continued) ^{1}H NMR of polymethoxyflavones isolated from citrus species

Name[a]	H-3	H-5	H-6	H-8	H-2′	H-3′	H-5′	H-6′	OMe	Ref
3,5,6,8,3',4'- Hexa-MF (12)					7.90 (d) (J = 9.0)		7.02 (d) (J = 9.0)	7.91 (dd) (J = 2.1, 9.0)	4.13, 4.04, 4.00, 4.00, 3.97, 3.96	*(40)*
3,5,6,7,3',4'- Hexa-MF (13)				6.75 s	7.70 (d) (J = 2.0)		6.99 (d) (J = 9.0)	7.71 (dd) (J = 9.0)	3.87, 3.92, 3.97, 3.98, 4.01	*(50)*
3,6,7,8,4'- Penta-MF (*Auranetin)* (14)		7.38			8.08	6.98	6.98	8.08		*(60)*
3,5,6,7,8,3',4'- Hepta-MF (15)					7.81 (d) (J = 2.0)		7.01 (d) (J = 8.0)	7.84 (dd) (J = 2.0, 8.0)	3.90, 3.95, 3.97, 3.97, 3.97, 4.00, 4.00	*(49)*
3-OH- 5,6,7,4' - Tetra-MF (17)				6.67 s	7.98 (d) (J = 9.0)	7.13 (d) (J = 9.0)	7.13 (d) (J = 9.0)	7.98 (d) (J = 9.0)	3.87, 3.84, 3.98, 3.85	*(27)*
5-OH- 6,7,8,4'- Tetra-MF (19)	6.61 s				7.91 (d) (J = 8.8)	7.05 (d) (J = 8.8)	7.05 (d) (J = 8.8)	7.91 (d) (J = 8.8)	4.13, 3.99, 3.97, 3.91	*(34)*
5-OH- 6,7,8,3',4'- Penta-MF (21)	6.61 s				7.42 (d) (J = 2.1)		6.99 (d) (J = 9.0)	7.58 (dd) (J = 2.1, 9.0)	4.12, 3.99, 3.99, 3.97, 3.96	*(40)*
7-OH- 5,6,8,3',4'- Penta-MF (24)	6.63 s				7.42 (d) (J = 2.0)		6.99 (d) (J = 9.0)	7.57 (dd) (J = 9.0, 2.0)	4.14, 4.04, 4.00, 3.98, 3.97	*(28)*
3-OH- 5,6,7,8,3',4'- Hexa-MF (25)					7.90 (d) (J = 2.0)		7.03 (d) (J = 9.0)	7.91 (dd) (J = 9.0, 2.0)	4.11, 4.03, 3.98, 3.97, 3.96, 3.95	*(28)*
5-OH- 3,6,7,8,3',4'- Hexa-MF (26)					7.66 (d) (J = 2.2)		7.08 (d) (J = 8.7)	7.75 (dd) (J = 2.2, 8.7)	3.88, 3.84, 3.83, 3.78, 3.74	*(38)*
7-OH-3,5,6,8,3',4'- Hexa-MF (27)					7.90 (d) (J = 9.0)		7.02 (d) (J = 9.0)	7.91 (dd) (J = 2.1, 9.0)	4.13, 4.04, 4.00, 4.00, 3.97, 3.96	*(40)*
5,7-OH- 6,8,3',4'- Tetra-MF (28)	6.60 s				7.40 (d) (J = 2.4)		7.10 (d) (J = 8.5)	7.52 (dd) (J = 8.5, 2.4)	4.10, 3.99, 3.97, 3.94	*(36)*

[a] The structures are given in Fig 1 and Fig 2 which correspond to the numerical given in brackets.

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Table 3. ^{13}C NMR of polymethoxyflavones isolated from citrus species

Name[a]	C															OMe							Ref
	2	3	4	5	6	7	8	9	10	1'	2'	3'	4'	5'	6'	6	7	8	5	3	3'	4'	
5,7,4'- Tri-MF (1)	162.1	107.1	177.7	159.9	96.1	163.9	92.8	160.7	109.3	123.9	127.6	114.4	160.9	114.4	127.6		55.7		56.4			55.5	*(49)*
3,6,7,4'- Tetra-MF (2)	160.3	106.1	175.6	97.3	139.8	161.8	157.4	153.9	114.5	123.0	127.8	114.5	151.6	114.5	127.8	55.5	61.8			61.0		56.4	*(27)*
5,6,7,4'- Tetra-MF (3)	162.0	106.8	176.8	154.3	140.5	157.5	96.2	152.5	112.4	123.7	127.4	114.2	160.9	114.2	127.4	61.3	56.1		61.9			55.3	*(40)*
5,7,8,4'-Tetra-MF (4)	162.3	106.9	177.9	152.0	92.6	156.3	130.8	156.3	109.1	123.8	127.7	114.5	160.7	114.5	127.7		56.2	61.5	56.6			55.0	*(40)*
5,6,7,8,4'- Tangeretin (5)	162.2	106.7	177.3	144.0	138.1	151.3	148.4	147.7	114.8	123.8	127.8	114.4	161.1	114.6	127.6	62.1	61.9	61.7	62.3			55.5	*(40)*
5,6,7,3',4'- Sinensitin (6)	160.0	107.4	177.2	154.5	140.4	157.6	96.3	152.6	112.9	124.1	108.7	149.3	151.9	111.0	119.7	61.6	56.3		62.2		56.1	56.0	*(40)*
5,7,8,3',4'- Penta-MF (7)	160.5	107.2	177.9	152.0	92.6	156.3	130.7	156.3	109.1	124.1	108.7	149.2	151.5	111.0	119.6		56.3	61.5	56.5		56.1	56.0	*(40)*
6,7,8,3',4'- Penta-MF (8)	160.3	106.4	175.7	97.3	139.7	157.4	151.5	153.9	111.7	123.2	109.2	149.0	151.7	112.0	119.4	55.7	61.8	55.9			61.0	56.4	*(27)*
5,6,7,8,3',4'- Nobiletin (9)	161.0	106.7	177.4	144.0	138.0	151.4	138.0	147.7	114.8	124.0	108.7	149.3	151.9	111.0	119.6	62.0	61.8	61.7	62.3		56.1	56.0	*(49)*
3,5,6,7,8,4'- Hexa-MF (10)	151.3	140.7	174.0	143.9	138.0	153.5	138.0	148.2	115.2	123.4	130.0	114.2	161.5	114.2	130.0	61.9	61.7	58.0	62.1	62.4		55.0	(28)
3,5,7,8,3',4'- Hexa-MF (11)	150.8	140.8	174.2	152.2	92.4	156.4	130.4	156.3	109.4	123.6	110.9	148.7	150.9	111.0	121.8		56.4	59.9	56.5	61.4	56.0	55.9	*(49)*
3,5,6,8,3',4'- Hexa-MF (12)	150.7	142.9	171.8	143.6	147.7	146.9	137.4	146.9	111.7	123.9	110.6	149.0	151.7	111.3	121.1	61.8		61.7	62.3	61.6	56.0	55.9	*(40)*
3,5,6,7,8,3',4'- Hepta-MF (15)	151.1	140.8	173.9	143.9	137.8	151.3	137.8	148.2	115.1	123.5	110.9	148.8	153.0	111.0	121.9	61.8	61.7	59.9	61.9	62.3	56.0	55.9	*(49)*

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Table 3. (Continued) ^{13}C NMR of polymethoxyflavones isolated from citrus species

Name[a]	C															OMe							Ref
	2	3	4	5	6	7	8	9	10	1'	2'	3'	4'	5'	6'	6	7	8	5	3	3'	4'	
5-OH,6,7,4'- Tri-MF (16)	163.6	103.3	182.3	152.0	131.9	158.7	91.6	152.7	105.1	122.7	128.3	114.6	162.4	114.6	128.3	60.6	56.5					55.6	*(51)*
3-OH,5,6,7,4'-Tetra-MF (17)	142.6	137.6	171.0	151.0	139.3	157.5	96.9	153.0	110.0	123.5	128.8	114.0	160.1	114.0	128.8	61.1	56.5		61.9			55.3	*(51)*
5-OH,3,7,3',4'-Tetra-MF (18)	155.5	138.3	178.1	160.9	97.9	165.2	92.5	156.4	105.3	122.1	111.6	148.5	151.4	111.3	122.1		56.2			59.8	55.7	55.7	*(51)*
5-OH,6,7,8,4'-Tetra-MF (19)	163.0	104.0	183.3	149.8	136.8	153.2	133.2	146.0	107.2	123.7	128.3	114.9	164.3	114.9	128.3	62.4	62.0	61.4				55.8	*(34)*
3-OH,5,6,7,8,4' -Penta-MF (20)	142.9	137.8	171.2	147.0	143.1	150.7	137.4	146.2	112.3	123.5	128.8	114.2	160.3	114.2	128.8	61.4	62.0	61.6	61.8			55.3	*(51)*
5-OH,6,7,8,3',4'-Penta-MF (21)	158.8	106.5	179.3	149.1	136.2	152.9	132.9	142.9	107.5	123.7	114.6	145.6	149.0	110.5	121.6	62.1	61.7	61.2			56.1	56.0	*(40)*
5-OH,3,7,8,3',4'-Penta-MF (22)	155.2	138.0	178.3	156.4	95.8	158.2	128.8	147.8	104.5	122.2	111.7	148.4	151.3	110.9	121.9		56.5	61.0		59.7	55.3	55.7	*(51)*
7-OH,3,5,6,3',4'-Penta-MF (23)	151.8	140.0	171.4	137.4	142.8	158.2	96.1	153.7	109.8	123.8	110.6	148.9	150.5	111.0	120.8		56.4		61.6	62.3	56.1	56.0	*(40)*
7-OH,5,6,8,3',4'-Penta-MF (24)	161.4	106.7	177.3	145.1	140.1	140.3	138.1	145.6	114.2	124.2	108.7	149.4	152.0	111.3	119.7	62.1		61.5	62.8		56.5	56.0	(28)
5-OH-3,6,7,8,3',4'-Hexa-MF (25)	155.6	138.1	178.7	148.5	135.5	152.5	132.4	144.4	106.8	122.1	110.9	148.1	151.4	111.8	122.0	60.6	61.8	61.5		59.8	55.4	55.7	*(51)*
7-OH,3,5,6,8,3',4'-Hexa-MF (26)	150.7	142.9	171.8	143.6	147.7	146.9	137.4	146.9	111.7	123.9	110.6	149.0	151.7	111.3	121.1	61.8		61.7	62.3	61.6	56.0	55.9	*(40)*
5,7-OH, 6,8,3',4'-Tetra-MF (27)	160.2	106.8	177.4	150.0	134.0	148.9	137.5	144.5	110.4	123.7	114.6	145.5	149.5	110.5	121.5	62.3		61.7			56.1	56.0	*(35)*

[a] The structures are given in Fig 1 and Fig 2 which correspond to the numerical given in brackets.

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APPENDIX B

Isolation and Chemical Characterization of Components with Biological Activity Extracted from *Azadirachta indica* and *Melia azedarach* (Chapter 4)

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In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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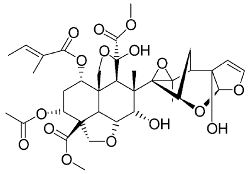
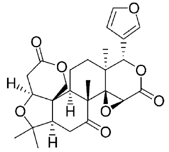
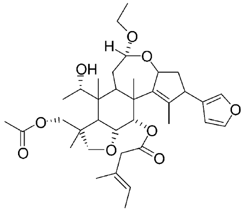
Table 1. Limonoids isolated from Melia azedarach



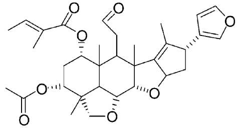
[1] azadirachtin A



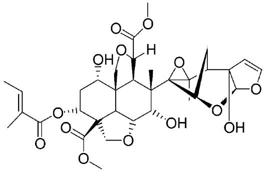
[2] limonin



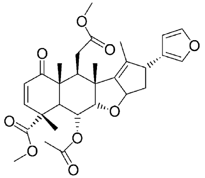
[3] 12-O-ethyl-1-deacetylnimbolinin B
Xie F. et al., 2008



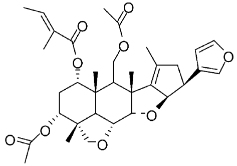
[4] 1-tigloyl-1-O-debenzoylohchinal
Xie F. et al., 2008



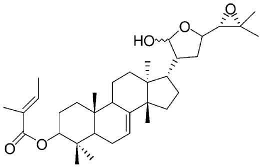
[5] azadirachtin B
Caboni P. et al., 2006



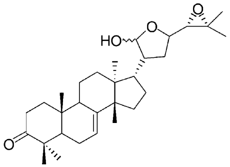
[6] nimbin
Caboni P. et al., 2006



[7] salannin
Caboni P. et al., 2006, Huang et al1994



[8] 3-α-O-tigloylmelianol
Ntalli N. et al. 2010



[9] melianone
Ntalli N. et al. 2010

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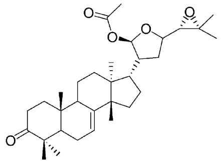
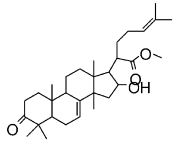
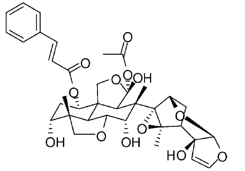
Table 1. (Continued) Limonoids isolated from Melia azedarach



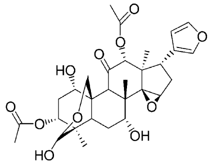
[10] 21-β-acetoxymelianone
Ntalli N. et al. 2010



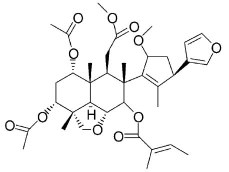
[11] methyl-kulonate
Chiang and Chang, 1973; Huang, 1999



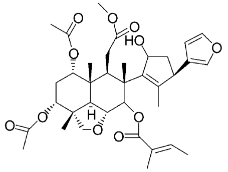
[12] 1-cinnamoyl-3,11-dihydroxymeliacarpinin
Alche et al., 2002



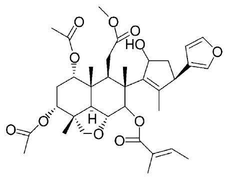
[13] 28-deacetylsendanin
Kim, M. et al. 1999



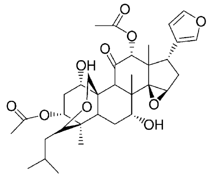
[14] 15-O-deactyl-15-O-methylnimbolidin A
Zhou H., et al. 2005



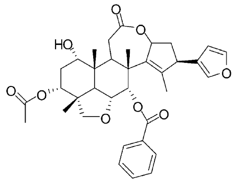
[15] 15-O-deacetyl-15-O-methylnimbolidin B
Zhou H., et al. 2005



[16] 15-O-deacetyl-nimbolidin B
Zhou H., et al. 2005



[17] 12-O-deacetyltrichilin H
Zhou H., et al. 2005



[18] 1-O-deacetylohchinolide A
Zhou et al., 2004

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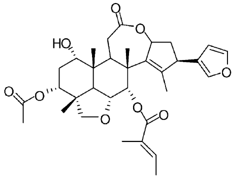
Table 1. (Continued) Limonoids isolated from Melia azedarach


O
HO
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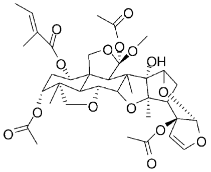
O
O

[18] 1-O-deacetylohchinolide B
Zhou et al., 2004


O
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OH
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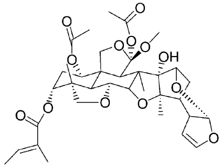
[19] 1-tygloyl-3,20-diacetyl-11-methoxymeliacarpinin
Takeya et al., 1996 [a]


O
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OH
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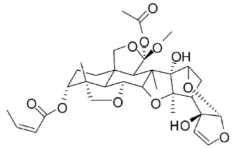
[20] 3-tigloyl-1,20-acetyl-11-methoxymeliacarpinin
Takeya et al., 1996 [a]


O
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OH
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HO
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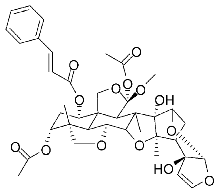
[21] 1-deoxyl-3-methacrylyl-11-methoxymeliacarpinin
Takeya et al., 1996 [a]


O
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O
OH
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O
O
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HO
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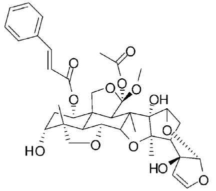
[22] 1-cinnamoyl-3-acetyl-11-methoxymeliacarpinin
Takeya et al., 1996 [a]


O
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O
OH
HO
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HO
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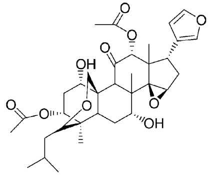
[23] 1-cinnamoyl-3-hydroxyl-11-methoxymeliacarpinin
Takeya et al., 1996 [a]


O
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OH
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OH
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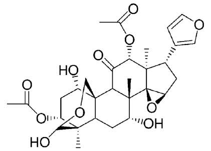
[24] 29-isobutylsendanin
Kipassa N., 2008


O
O
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HO
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O
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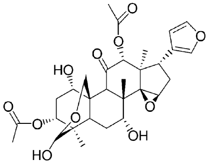
O
OH
HO
O

[25] 12-hydroxyamoorastatin
Nakatani et al., 1998, Carpinella et al., 2003,


O
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O
HO
O
O
O
O
OH
HO

[26] 29-deacetylsendanin or toosendanin
Kipassa N., 2008

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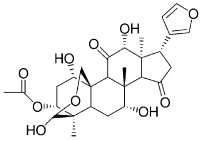
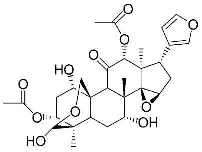
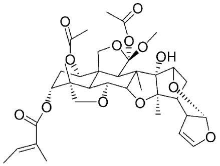
Table 1. (Continued) Limonoids isolated from Melia azedarach



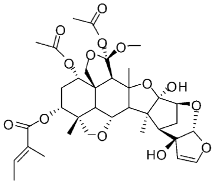
[27] 12-hydroxyamoorastatin
Kipassa N., 2008



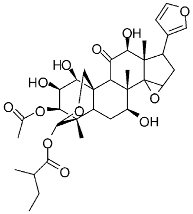
[28] 12-acetoxyamoorastatin
Kipassa N., 2008



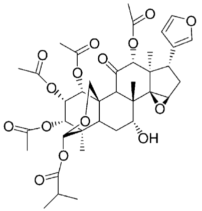
[29] 1-tygloyl-3-acetyl-1-methoxymeliacarpinin
Itokawa H., 1995



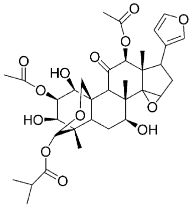
[30] 1-acetyl-3-tigloyl-11-methoxymeliacarpinin
Itokawa H., 1995



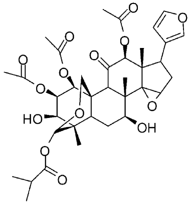
[31] 12-deacetyltrichilin I
Takeya et al., 1996 [b]



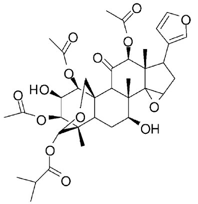
[32] 1-acetyltrichilin I
Takeya et al., 1996 [b]



[33] 3-deacetyltrichilin H
Takeya et al., 1996 [b]



[34] 1-acetyl-3-deacetyltrichilin H
Takeya et al., 1996 [b]



[35] 1-acetyl-2-deacetyltrichilin H
Takeya et al., 1996 [b]

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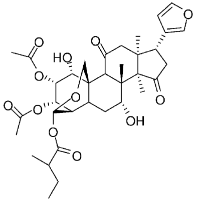
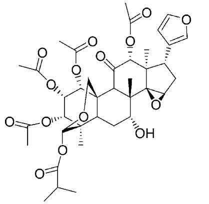
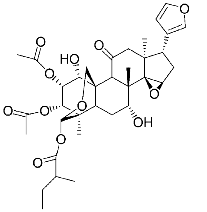


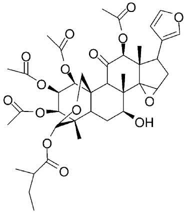
[36] meliatoxin B1
Huang et al., 1994; Takeya et al., 1996



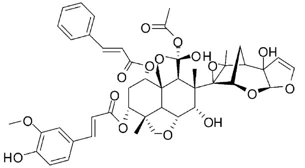
[37] trichilin H
Huang et al., 1994; Takeya et al., 1996 [b]



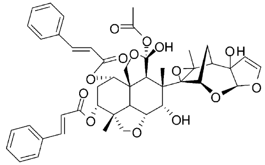
[38] trichilin D
Takeya et al., 1996 [b]



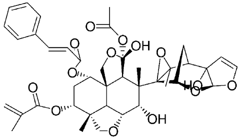
[39] 1,12-di-O-acetyltrichilin B
Takeya et al., 1996 [b]



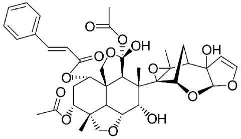
[40] 11-α-hydroxy-1-cinnamoyl-3-feruloyl-meliacarpin
Bohnenstengel et al., 1999; Ayyad et al, 2008



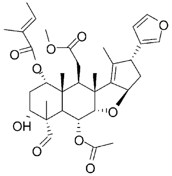
[41] 1,3-dicinnamoyl-11-hydroxymeliacarpin
Bohnenstengel et al., 1999



[42] 1-cinnamoyl-3-methacrylyl-11-hydroxy-meliacarpin
Bohnenstengel et al., 1999



[43] 1-cinnamoyl-3-acetyl-11-hydroxymeliacarpin
Bohnenstengel et al., 1999



[44] salannal
Huang et al., 1996

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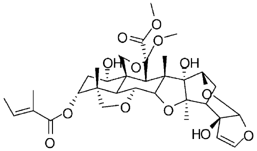
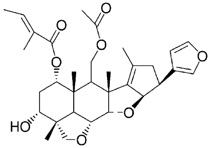
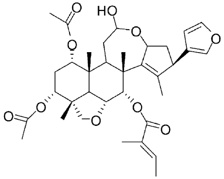
Table 1. (Continued) Limonoids isolated from Melia azedarach



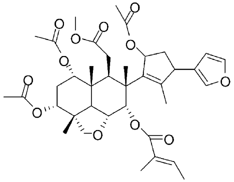
[45] meliacarpinin E
Huang et al., 1996



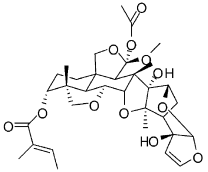
[46] deacetylsalannin
Huang et al., 1996



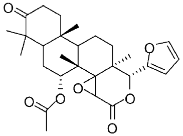
[47] nimbolinin B
Huang et al., 1996



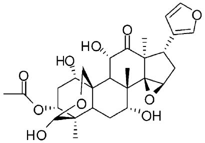
[48] nimbolidin B
Huang et al., 1996



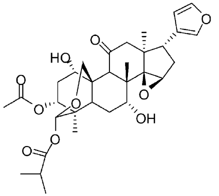
[49] 1-deoxy-3-tigloyl-11-methoxymeliacarpinin
Kipassa N., 2008, Nakatani et al., 1995; Huang et al., 1994



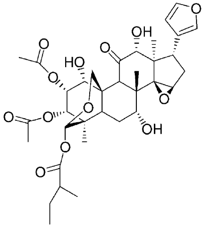
[50] gedunin
Huang, 1999Kipassa N., 2008



[51] meliartenin
Carpinella et al., 2002, 2003



[52] azedarachin C
Huang et al., 1995



[53] trichilin B
Huang et al., 1994

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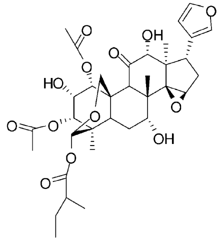
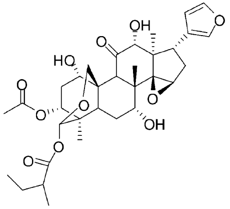
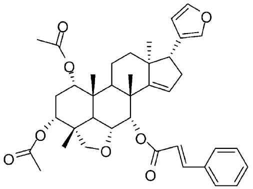
Table 1. (Continued) Limonoids isolated from Melia azedarach



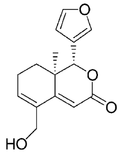
[55] azedarachin A
Huang et al., 1994



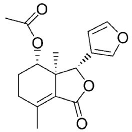
[56] nimbolin A
Huang et al., 1994



[57] nimbolin A
Huang et al., 1994

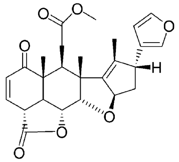


[57] azedaralide
Nakatani et al., 1998; Okamura et al., 1997
Fukuyama et al., 2006

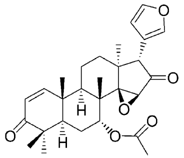


[58] 12α-acetoxyfraxinellone
Nakatani et al., 1998; Okamura et al., 1997
Fukuyama et al., 2006

[59] fraxinellone
Nakatani et al., 1998; Okamura et al., 1997
Fukuyama et al., 2006



[60] nimbolide
Kipassa N., 2008



[61] epoxyazadiradione
Kipassa N., 2008

APPENDIX C

Chemical Composition and Biological Effects of Maple Syrup (Chapter 18)

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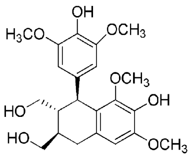
In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.



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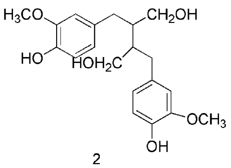

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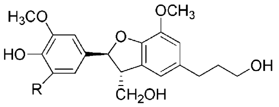
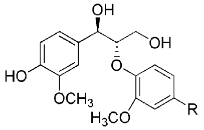
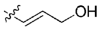
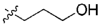
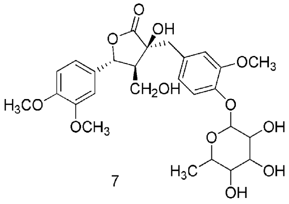


3 R= H
4 R= OCH$_3$



5 R= 
6 R= 



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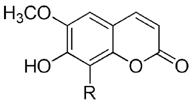
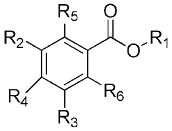
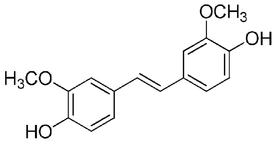


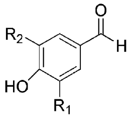
8 R= H
9 R= OH



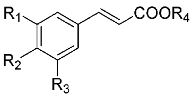


10

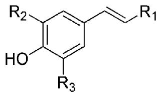
11 R_1=R_5=R_6=H R_2=R_3=R_4= OH
12 R_1= CH_3 R_2=R_3=R_4= OCH_3 R_5=R_6=H
13 R_1=R_4=OH R_2=R_3= OCH_3 R_5=R_6=H
14 R_1=R_3=R_5=R_6=H R_2=R_4=OH
15 R_1=R_2=R_5=R_6=H R_3=OCH_3 R_4=OH
16 R_1=R_2=R_3=R_4=R_5=R_6=H
17 R_1=glucose R_2=R_3=R_4=OH R_5=R_6=H
18 R_1=R_2=R_3=R_4=H R_5=R_6=OH
19 R_1=R_2=R_4=R_6=H R_3=R_5=OH



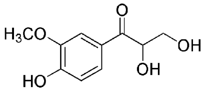
20 R_1= H R_2= OH
21 R_1=H R_2= OCH_3
22 R_1=R_2= OCH_3



23 R_1=R_3=R_4=H R_2=OH
24 R_1= OCH_3 R_2= OH R_3=R_4= H
25 R_1=R_3=R_4=H R_2=OCH_3
26 R_1=R_3=OCH_3 R_2=OH R_4=H
27 R_1=R_2=OH R_3=H R_4=quinic acid



28 R_1= CH_2OH R_2=R_3= OCH_3
29 R_1= CH_2OH R_2= OCH_3 R_3= H



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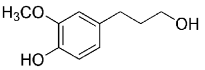

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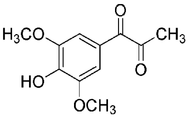

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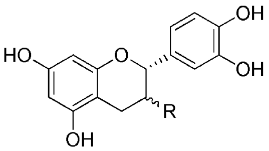
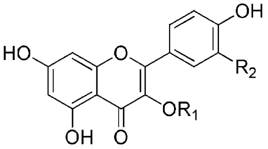
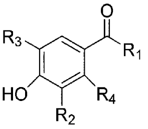


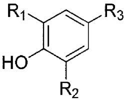
33 R= ▬OH
34 R= ┄OH


HO
OH
R2
OR1
OH O

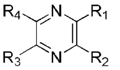
35 R_1=glucose R_2=H
36 R_1=galactose R_2=H
37 R_1=glucose R_2=OH
38 R_1=rhamnose R_2=OH
39 R_1=rutinose R_2=OH

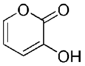

O
R3
R1
HO
R4
R2

40 R_1= CH_2OH R_2= OH R_3=R_4= H
41 R_1=R_3= CH_3 R_2=R_4= OH
42 R_1= CH_3 R_2= H R_3=R_4= OH
43 R_1=CH_3 R_2=R_3=OH R_4=H



44 R_1=OH R_2=R_3=H
45 R_1=R_2=OCH_3 R_3=H
46 R_1= OCH_3 R_2= H R_3=CH_2COOH


R4
N
R1
R3
N
R2



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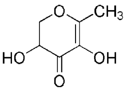

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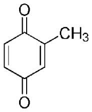
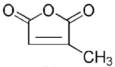


58

47 R_1=R_4=CH_3 R_2=R_3=H
48 R_1=CH_3 R_2=R_3=R_4=H
49 R_1=R_3=CH_3 R_2=R_4=H
50 R_1=R_2=CH_3 R_3=R_4=H
51 R_1=R_3=CH_3 R_2=R_4=isobutanol
52 R_1=CH_2CH_3 R_2=R_3=R_4=H
53 R_1=CH_2CH_3 R_2=R_3=H R_4=CH_3
54 R_1=n-butyl R_2=R_3=R_4=H
55 R_1=isopropyl R_2=H R_3=R_4=CH_3



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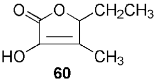
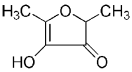
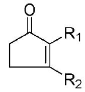

O
O
CH2CH3
HO
CH3

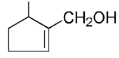
60


H3C
O
CH3
HO
O

61



62 R_1=CH_3 R_2=H
63 R_1=CH_2OH R_2=H
64 R_1=OH R_2=CH_3



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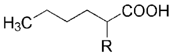
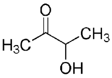


66 R=H
67 R=CH_2CH_3



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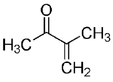
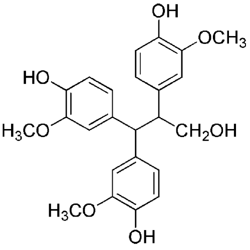

O
H3C
CH3
CH2

69


OH
OCH3
HO
H3CO
CH2OH
H3CO
OH

70

Figure 1. Structures of chemical constituents identified in maple syrup. Compounds 1- 46, and 70 are phenolics and compounds 47-69 are non-phenolic constituents.

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APPENDIX D

Urinary Pharmacokinetics of Queen Garnet Plum Anthocyanins in Healthy Human Subjects (Chapter 21)

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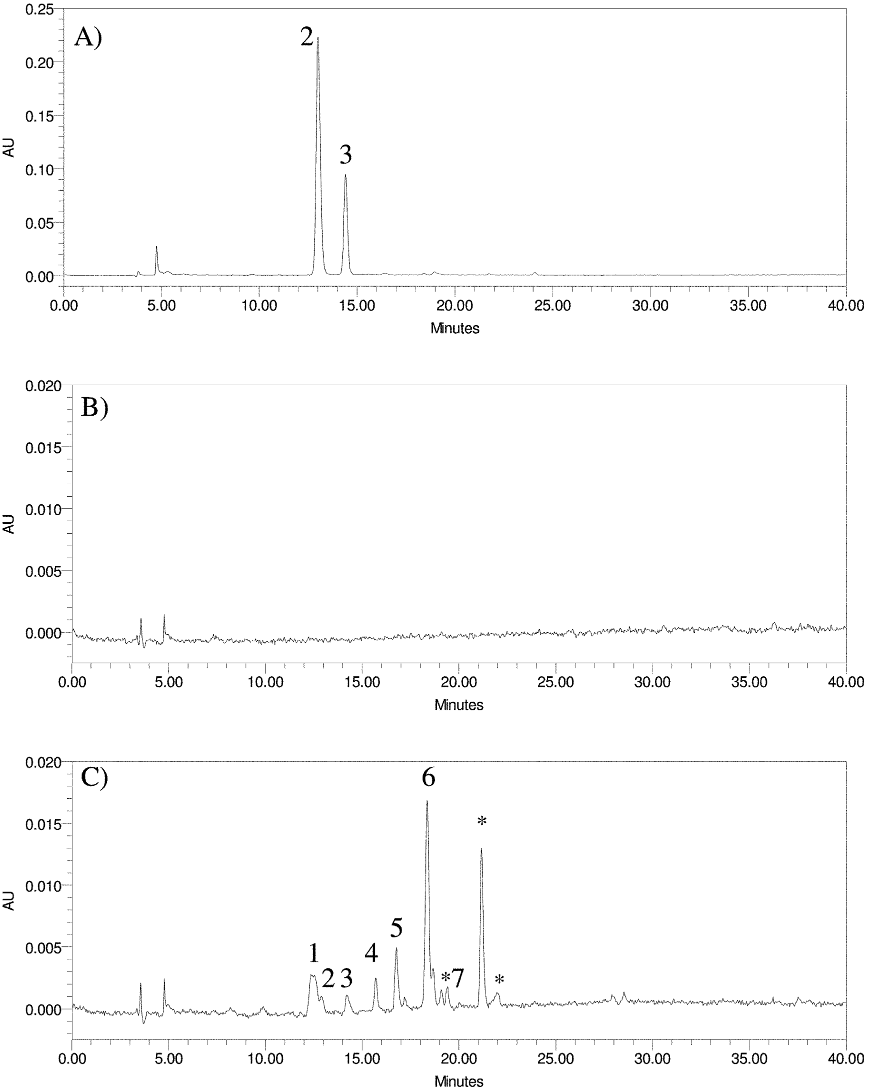
In Emerging Trends in Dietary Components for Preventing and Combating Disease; Patil, B., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.



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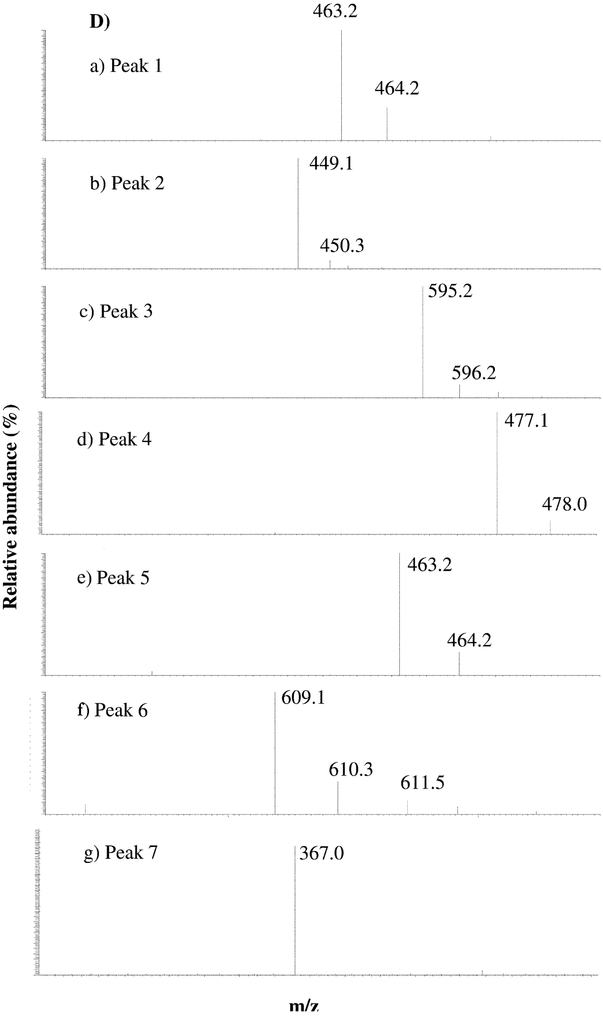

Figure 3. Representative HPLC chromatograms (A-C) and fragmentation patterns (D) of QGPJ (A) and of human urine (one subject) before (B) and 4 h after (C) the consumption of 400 mL QGPJ. Detection was performed at 520 nm. Urine was treated by solid phase extraction. Peaks: (1) cyanidin monoglucuronide, (2) cyanidin-3-glucoside, (3) cyanidin-3-rutinoside, (4) peonidin monoglucuronide, (5) peonidin-3-glucoside, (6) peonidin-3-rutinoside, (7) cyanidin monosulfate, and (*) unknown anthocyanin metabolites. (D) HPLC-ESI-MS precursor scans for product m/z 287 (a-c, g) and m/z 301 (d-f) showing native QGP anthocyanins and metabolites with intact flavylium skeleton. Components are numbered according to (C).

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